PATHOLOGICAL TECHNIQUE

A Practical Manual for Workers in Pathological Histology and Bacteriology

including

Directions for the Performance of Autopsies and for Clinical Diagnosis by Laboratory Methods

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TO

HENRY F. SEARS, A. M., M. D.,

WHO BY HIS LIBERALITY FIRST RENDERED POSSIBLE PATHOLOGICAL RESEARCH IN BOSTON, AND BY HIS PERSONAL WORK ADVANCED AND STIMULATED IT,

THIS BOOK IS RESPECTFULLY DEDICATED BY

THE AUTHORS

PREFACE TO THE SIXTH EDITION.

THIS edition, like its predecessors, contains a number of additions, partly of standard methods which have not been incorporated earlier because they seemed of less use to the pathologist than to the histologist, partly of new methods which have recently appeared. Bielschowsky's silver impregnation stain for nerve-fibers and for connective-tissue fibrils and reticulum has proved of great value in certain lines of pathological work. Bensley's excellent methods for the demonstration of mitochondria and other cytoplasmic granules, especially in the cells of the pancreas, should prove helpful in many ways. G. Herxheimer's alcohol-acetone solution of Scharlach R. for staining fat offers decided advantages over the solution heretofore in use, giving better results and acting more quickly.

Other additions are the complement-fixation test for gonorrheal infection, Lange's colloidal gold test for syphilis of the central nervous system, the complement-fixation test for echinococcus cyst, and Eyenes and Sternberg's silver impregnation method for staining the Treponema pallidum in sections. Short sections, descriptive of the bacillus of pertussis, of the Blastomyces, and the Sporothrix schenckii have also been inserted.

Many good books upon the several technical lines of work which we attempt to cover are now on the market. In this,

as in former editions, it has been deemed advisable to present but a limited number of the more useful and reliable methods, rather than to attempt a description of all available ones.

BOSTON, MASS., *April*, 1915.

PREFACE TO THE FIRST EDITION.

THIS book is designed especially for practical use in pathological laboratories, both as a guide to beginners and as a source of reference for the advanced. We believe that the book will also meet the wants of practitioners who have more or less opportunity to do general pathological work.

Every autopsy presents for solution a problem which may be simple or complex. The known quantities are certain clinical symptoms and physical signs; the unknown quantities are not only the gross and microscopic lesions that may or may not have given rise to the symptoms and signs, but also the etiology of the lesions and the order of their sequence. The solution of the problem often requires the highest skill in post-mortem, bacteriological, and histological technique, but in its solution lies the fascination of pathological work.

It has seemed advisable to us to present, so far as possible, a consecutive statement of the methods employed in solving the various problems that arise, so as to avoid the repetitions that necessarily occur when the three usual divisions of the subject are separately considered by different writers. It is hoped that this method of presenting the subject will bring the student to the realization that the mechanical performance of a post-mortem examination and the inspection of the gross lesions constitute usually only the beginning of the solution of the problem, which should be investigated

bacteriologically, histologically, and chemically as far as our present knowledge will permit.

We should particularly advise the routine bacteriological and histological examination of the more important organs in all suitable cases. Naturally, the autopsies in which the lesions are due to a single etiological factor are the most valuable and instructive for a clear understanding of the pathological processes present.

Besides the methods of post-mortem examinations and of bacteriological and histological investigations connected with autopsies, we have added the special methods employed in clinical bacteriology and pathology.

In the parts devoted to Bacteriology and to Pathological Histology we have not endeavored to make an exhaustive collection of methods and formulæ, but rather to select those which have been found of the greatest service in practical work.

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PART I.

POST-MORTEM EXAMINATIONS.

Introduction.—The method of making post-mortem examinations most generally followed in this country and abroad is that originally taught by Virchow. It has been variously modified in minor details by his pupils and followers. The strongest adverse criticism which can be made of the method is that it works best when the various organs in the body are nearest normal. Its chief fault lies in the early separation of the different structures from each other, so that interesting pathologic relations are often overlooked and destroyed. Special procedures are advised for certain conditions, but emphasis is not placed on them.

Two other methods of making post-mortem examinations were developed besides that of Virchow's, but have never attained the same general recognition, although both contain principles of great value.

The technique of Rokitansky, now best exemplified by Chiari, appeared in printed form a number of years ago. Its fundamental principle is to examine and open every organ *in situ* before removing it, so that all abnormal relations between organs, blood-vessels, and other structures can be discovered and preserved intact.

Recently, Hauser has published Zenker's post-mortem technique. Its main principle is to remove the organs in

block, so that they can be examined from every side and incised before they are separated from each other.

In both methods the incisions in the various organs, and especially the heart, differ more or less radically from those recommended by Virchow.

A knowledge of all three forms of procedure is useful. It broadens one's point of view, and tends to keep one's mind open to possibilities. A list of the best publications on post-mortem technique is appended at the end of this section of the book.

The problem offered by an autopsy is often solved in part or wholly by the macroscopic post-mortem examination. More frequently, however, the complete and final solution is reached only after careful bacteriological and histological study. The post-mortem examination may, therefore, be looked upon as the beginning of the solution of the problem. Its particular function is to demonstrate in the individual case all congenital or acquired abnormalities, all macroscopic lesions, and to explain all gross mechanical questions. It furnishes the material for bacteriological and histological study. Perfectly to accomplish its purpose a post-mortem examination must be made in a careful, systematic manner.

While a general method of procedure is advisable, it will often be found advantageous, or even necessary, to depart from it. According to Orth, "the chief requisite of every exact post-mortem examination is this, that no part shall be displaced from its position until its relations to the surrounding parts are established, and that no part shall be taken out by whose removal the further examination of other parts is affected."

The order and method of procedure in making a post-mortem examination, including the various incisions, may be said to have been planned for the routine examination of normal or diffusely diseased organs. As soon as a noticeable focal lesion is present the order of procedure and the customary method of removal and of incision must be so altered as best to display the lesion.

Instruments.—The following instruments will be found extremely useful in the autopsy-room, although not all of them are necessary:

The *autopsy-table* should be large, so as to accommodate on it the instruments and several dishes in addition to the body. It should have a slightly raised edge, and should slope gently toward an opening in the center for the escape of fluids. The table is best made of zinc, and along one edge should have a centimeter scale. The water for use on the table is best supplied by a rubber tube from an overhead pipe reaching to within 60 to 100 cm. of the table.

The *scales* for weighing the various organs should have a large pan and gram and kilogram weights.

A *band-saw* will be found very useful for sawing bones for the inspection of the marrow, and for calcified and osseous tumors.

The best *autopsy-knife* is a stout, broad-bladed knife with bellied edge and heavy handle. The blade should measure about 12 cm. in length and 3 cm. in width; the handle should be 12 cm. in length. Many operators prefer a somewhat smaller knife than this.

Amputating-knives of different sizes are useful for long, deep cuts into organs and tumors.

A *myelotome* is a short, thin, narrow knife-blade, 1.4 cm. long and 4 mm. wide, set obliquely on a slender steel stalk ending in a wooden handle (Fig. 2). It is used only for cutting the cord squarely across in removing the brain.

Cartilage-knives and *scalpels* of different sizes are useful for a variety of purposes.

Scissors, both straight and curved, should be of various sizes. A medium-sized and a fine pair should each have one probe-pointed blade.

An *enterotome* is a long, straight pair of scissors, of which one blade is longer than the other and blunt at the extremity (Fig. 3). A hook at the end is not advisable. The instrument is used in opening the heart and the intestines.

A *saw* with movable back and rounded end will be found the most generally useful for opening the skull and the

spinal canal. An ordinary meat-saw is preferred by some, but cannot be used on the vertebræ.

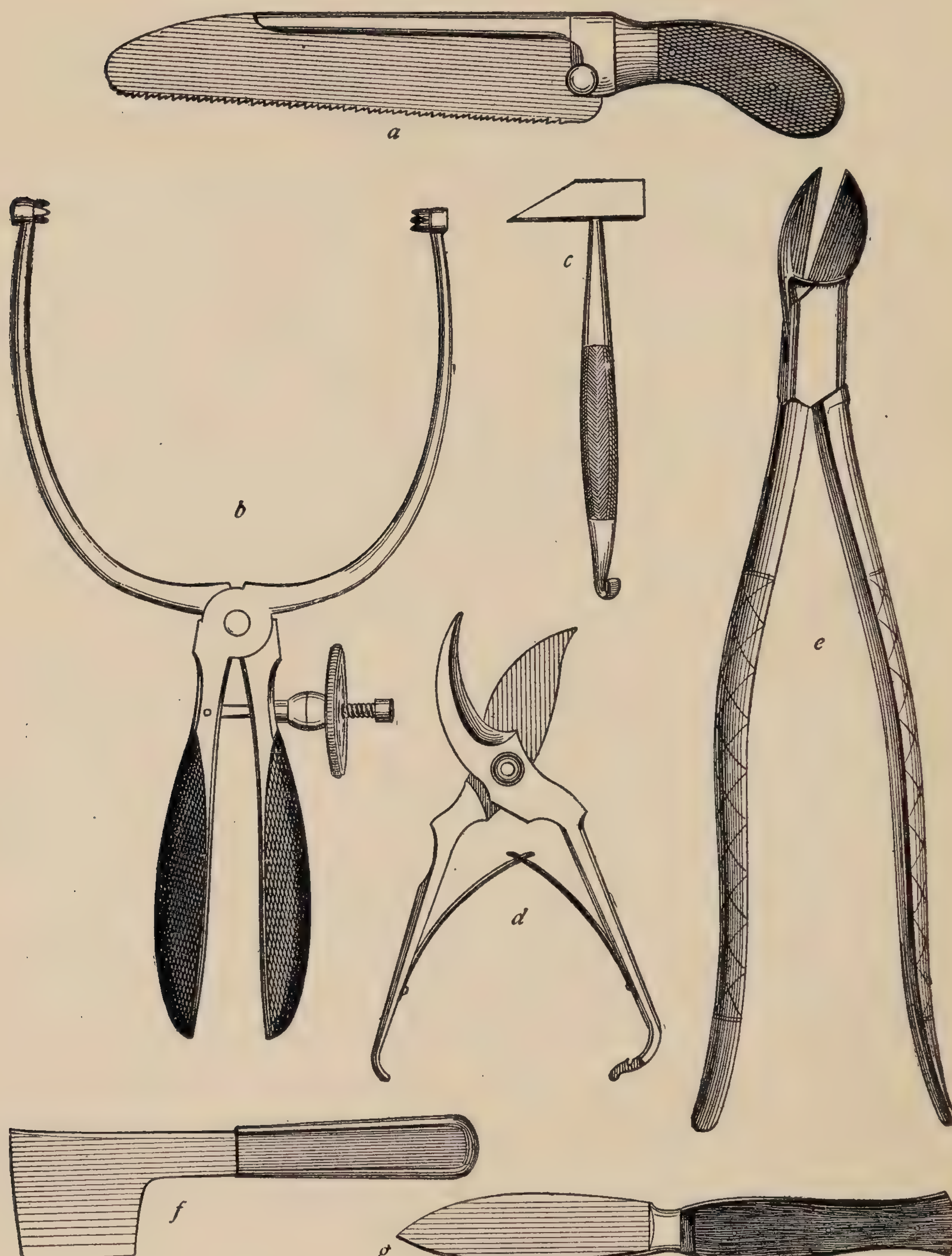

FIG. 1.—Instruments for use in the autopsy-room: *a*, saw; *b*, holder for the head; *c*, steel hammer with wedge end and blunt hook on the handle; *d*, costotome; *e*, bone-cutter; *f*, hatchet-chisel; *g*, autopsy-knife.

Luer's double rachiotome, or adjustable double saw (Fig. 4), is very useful in removing the cord, and is the safest instrument to put into the hands of beginners.

Forceps: several sizes, large and small, mouse-toothed.

Costotome: heavy bone-shears for cutting the ribs.

A powerful *bone-cutter*, with short blades, 5 cm. long, set at an angle of about 45° to the handles, which are 36 cm.

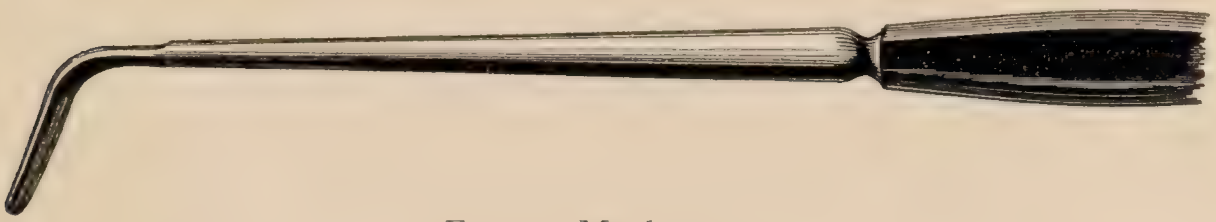

FIG. 2.—Myelotome.

in length, is employed for dividing the arches of the cervical vertebræ and for other purposes where ordinary bone-cutters will not do.

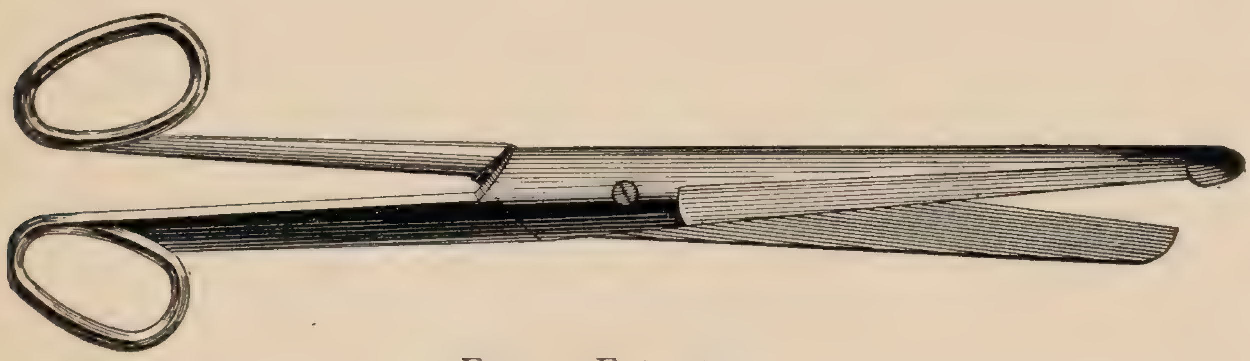

FIG. 3.—Enterotome.

A *chisel* with 2 cm. cutting edge, for exposing the marrow of the long bones, removing portions of the base of the skull, etc.

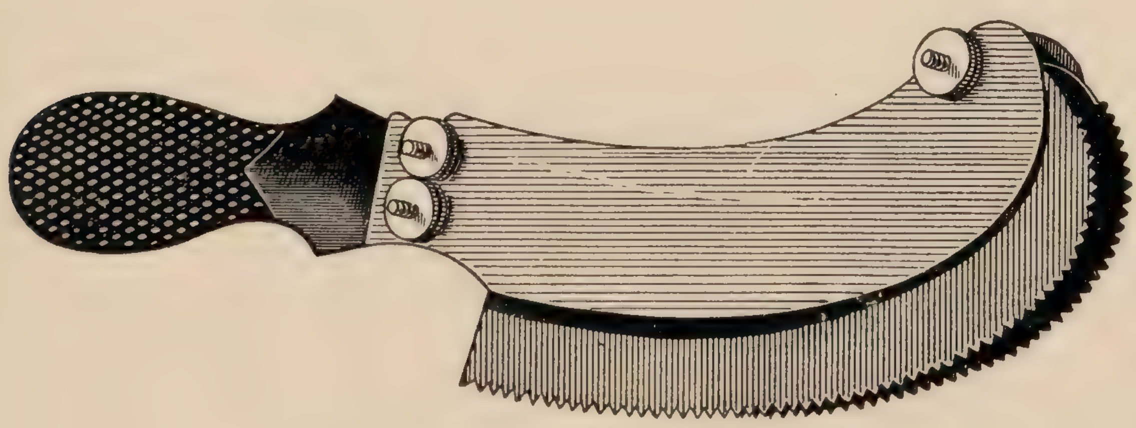

FIG. 4.—Luer's double rachiotome.

A *hatchet-chisel* of steel for starting the calvaria and spinous processes after sawing the skull and the vertebral column.

Soft-iron hammer with wooden handle.

Steel hammer with wedge end, and blunt hook on the handle.

Holder for the head while sawing the skull.

Autopsy-needles, long and a little curved.

Probes of flexible metal; also fine glass probes for small blood-vessels or ducts.

Grooved director.

Pans for holding water, organs, etc.

Boards, square or oblong, 30×30 or 30×50 cm., on which to lay instruments or cut organs.

Sponges.

Catheters.

Strong *hemp twine* is the best for sewing up the body.

Glass graduates for measuring fluids.

A *block of wood* with shallow depression for the neck; for use while opening the head.

Vise.

Small *cup* or *dish* for removing fluid from cavities.

General Rules.—The room for an autopsy should be well lighted, otherwise the finer changes in the tissues cannot be recognized. Artificial illumination is not good, because the colors of the tissues are entirely changed by the yellowness of the light.

Before beginning an autopsy the necessary instruments should be arranged on a short board on the autopsy-table in the order in which they are most likely to be used.

The operator stands on the right side of the body. This position he rarely leaves except for some definite purpose; for example, in opening the skull he stands at the head.

Order and cleanliness are the first points to be insisted upon at every autopsy. Clean water should always be at hand for washing the instruments and for keeping the hands free from blood and pus. The cut surface of an organ should not be washed with water except to remove blood; gently scrape the surface with the knife held obliquely.

In cutting, the knife should be drawn, not pressed or shoved into the tissues. According to Virchow, a broad, clean cut into an organ, even if incorrectly made, is much better than several short cuts which leave a ragged surface.

The autopsy-knife should be grasped in the hand as if to

cut bread. In using this knife the main movement should be from the shoulder, not from the wrist as in dissecting. It goes without saying that the sharper the knife the better.

In cutting the brain and cord, especially if their consistency is lessened, moisten the knife to prevent the tissue from sticking and tearing.

Before beginning an autopsy it is important to know the main points in the clinical history of the case, as they may greatly lighten the work of investigation by calling attention to those organs that require special examination.

The record of an autopsy should be dictated by the operator as he proceeds with the examination of the case, and should be as nearly as possible an objective description of the appearances found. Only the anatomical diagnosis should express the opinion of the operator. If it is not convenient to dictate the autopsy during its performance, the description of the lesions certainly ought to be made with the organs in sight, and not from memory after the lapse of hours or even days, when many of the details may be forgotten. Later, the results of the bacteriological and histological examinations should be added to the autopsy report, so as to make the case complete.

The thin rubber gloves now used by surgeons are very useful in making post-mortem examinations, especially in septic cases and while opening the stomach and intestines. Rubber cots for the fingers are often useful.

For cuts on the fingers use celloidin dissolved in equal parts of alcohol and ether, instead of flexible collodion, because the latter will not stick. A cut received during an autopsy should immediately be washed thoroughly. For protection during the rest of the autopsy, use a rubber glove or cover the cut with celloidin.

After an autopsy the operator should scrub his hands thoroughly with soap and brush, just as a surgeon does before an operation, and then use, if he so desires, an antiseptic solution, such as corrosive sublimate (1 : 2000) or 70 per cent. alcohol. For removing odors from the hands, turpentine will often be found serviceable, or a saturated solution of permanganate of potassium followed by oxalic acid.

For infections of slight wounds, such as scratches, or such as occur in hair-follicles, the best treatment within the first twenty-four hours is to bore into them with a sharp-pointed orange-wood stick dipped in strong carbolic acid, followed by washing with 95 per cent. alcohol. The procedure is practically painless, and the infection is stopped in the very beginning. Where the infection has spread, surgical treatment must be resorted to.

Hand Lotion.

Gum tragacanth,	10 grams;
Boric acid,	20 "
Glycerin (double distilled),	50 c.c.;
Water,	ad 1000 "
Oil of rose geranium,	1 "

Mix the first four ingredients in a bottle, and place in a warm place. Shake occasionally. After one to four days (whenever the gum is thoroughly softened) filter or squeeze through fine cheese-cloth or a towel, on the dry surface of which the oil of geranium has been poured. The amount of glycerin can be increased if desired.

Suggestions to Beginners.—In a case of *general miliary tuberculosis* the older focus from which the organisms have spread must always be found. Look especially for tubercular thrombi in the pulmonary veins as a frequent source of the general infection.

In a case of *embolism* hunt for the thrombus, bearing in mind, however, that the whole of a thrombus may become free and form an embolus. An arterial embolus may be due to a venous thrombus, in which case it must have passed through an open foramen ovale, except in the case of thrombi of the pulmonary veins.

In *acute peritonitis* always seek for a source of infection (appendix, female genitals, gastro-intestinal tract, etc.). It cannot always be found.

In *hemorrhage from the stomach* associated with cirrhosis of the liver look for rupture of dilated esophageal veins.

In cases of more or less *sudden death*, especially if preceded by signs of asphyxia, always examine the pulmonary artery *in situ* for possible emboli. In cases of *instantaneous death* examine the coronary arteries.

Private autopsies must often be made under many disadvantages, and, when out of town, not infrequently in a short space of time. It is always important to warn the attending physician not to allow the undertaker to inject the body before the autopsy, because the color and consistency of the organs are so changed by most injecting fluids that it is difficult to recognize the pathological processes. If there is danger of post-mortem changes, have the body packed in ice.

A regular autopsy-bag will be found very convenient for carrying to private autopsies. It is made of leather lined with rubber, and measures about 40 × 18 × 20 cm. Loose within it is carried a rubber bag 40 × 24 × 20 cm., shaped like a short envelope with a flap (22 cm. long) on one side, for bringing away any organs that demand further examination. The case of instruments should contain one or two autopsy-knives, two scalpels, a pair of forceps, one or two pairs of scissors, an enterotome, a steel hammer with wedge-end and with a blunt hook on the handle, a small chisel, a saw with detachable handle and back, an autopsy-needle, and a probe; free within the bag should be carried a spool of strong twine, a costotome, a long slender knife for use in removing the brain, a hammer with soft iron head, and a sponge. In rare cases additional instruments may be required. A white duck apron for personal use will always be found convenient. It is also well to carry along several blood-serum tubes and a platinum needle for making cultures at the autopsy. When there is a lesion of the nervous system it is advisable to bring a jar of a 4 per cent. solution of formaldehyde and to place the tissue in the fluid at the autopsy, as otherwise it is not easily gotten to the laboratory in good condition.

At the house can always be obtained a slop-pail, a wash-bowl, a pitcher of water, several newspapers, and an old sheet. The body is usually on an undertaker's frame, but

it may be in an ice-box or on the bed. The examination of the chest and abdomen can be made in any of these positions. If, however, the body is in an ice-box, it must be raised to the level of the top of the box in case it is necessary to open the head.

The clothing on the body can be removed, or, if only a shirt or a night-dress, is best slit down the middle and turned out over the arms. Tear the sheet into four equal pieces. Fold and tuck in one piece on each side of the trunk and neck, allowing the outer portion to fall over the arms. Fold and lay the third piece on the lower extremities, tucking the upper end beneath the clothing below the pubes. The fourth piece can be placed beneath the head if it is to be opened. This procedure leaves the front of the thorax and abdomen free for operation and protects the rest of the body and the clothing. On the thighs place one or two folded newspapers, and on these the necessary instruments. On the legs place the bowl containing only a dampened sponge. If the undertaker has not put a rubber sheet on the floor beneath the body and on the side where the operator is to stand, newspapers should be spread to protect the carpet. Place the slop-pail on the rubber sheet within convenient reach. Having thus made all arrangements, even to the threading of his needle, the operator is ready to begin.

If the cord and brain have to be examined as well as the body, it is best to do the cord first, so as to avoid the leakage that might otherwise occur from the trunk-cavities if they had been opened first. To support the head while opening it, use a stick of wood, a brick, or, in case of necessity, the instrument-box wrapped in a newspaper.

At a private autopsy cleanliness is extremely important. If there is no undertaker or nurse present, the operator himself must see that everything is cleaned and put in order before leaving, that all the blood-stains are removed from the dishes, and that all papers and soiled cloths are burned or rolled up and left in a neat bundle for the undertaker to dispose of. Ground coffee thrown on a shovelful of burning coals will be found helpful in disguising the odor in the room after an autopsy.

EXTERNAL EXAMINATION OF THE BODY.

External examination is often of great importance, especially in medico-legal autopsies, and should never be neglected, as it may throw great light on lesions found within the body. It should be systematic and careful, and is best taken up in the following order:

I. Inspection of the Body as a Whole.

1. Sex.

2. Age.

3. The *body-length* should be measured on the table beside the body, between points opposite the vertex of the head and the sole of the foot beneath the ankle.

4. The *development of the skeleton* has reference to the bony framework, which may be powerful, slender, or deformed.

5. The *general nutrition* is shown by the amount of muscular development and of subcutaneous fat-tissue. The latter is judged by pinching up folds of skin.

6. The *general condition of the skin* includes amount of elasticity, bronzing, jaundice, edema, and decubitus.

7. *Post-mortem discolorations* may be divided into three varieties:

(*a*) *Hypostasis of blood*, or the settling of blood into the lowest lying blood-vessels; this form of discoloration disappears on pressure.

(*b*) *Diffusion of blood-coloring matter* out of the vessels into the surrounding tissues (due to blood-pigment being set free by post-mortem decomposition); does not disappear on pressure.

(*c*) The *greenish discoloration*, usually seen earliest over the abdomen, is due to sulphide of iron formed through decomposition of the tissues. This discoloration is important, as it may modify the interpretation of appearances observed in the internal organs.

8. *Post-mortem rigidity*, degree and extent. It begins in the maxillary muscles, and spreads gradually from above downward, disappearing later in the same order. It is most marked and lasts longest in muscular individuals who have

been ill but a short time. Cholera furnishes the most marked cases. The rigor disappears quickest in cachectic diseases. When once it has been forcibly overcome, it does not recur. The time of beginning after death varies widely—from ten minutes to seven hours.

II. Special Inspection of the Different Parts of the Body.

The examination should begin with the head. Any lesion or abnormality found should be carefully noted. Particular attention should be paid to the condition of the pupils and to the color of the sclera. Then follow in order the neck, the thorax (size and shape), the abdomen (distended or retracted), the genitals, and the extremities.

INTERNAL EXAMINATION OF THE BODY.

The opening of the body-cavities is described first, because the brain is relatively much less frequently the seat of disease, and because in this country it is often impossible to obtain permission to open the head. Moreover, the lesions in the body often throw much light on those to be expected in the brain. The advantage of examining the brain first, particularly in those cases in which the important lesions are cerebral, is said to be that the amount of blood in the cerebral vessels can be more accurately determined. After the heart has been removed some of the blood in the brain may escape through the severed vessels below.

In routine examinations, however, the body is usually examined first, then the brain, and finally the cord. It is not a bad practice to remove the calvarium, to examine the meninges over the upper surface of the cerebrum, and then to make the examination of the body before removing the brain. In this way any change in the blood-supply of the cerebral vessels would be observed.

Opening of the Abdominal Cavity.—In the examination of the body the peritoneal cavity is opened first, the two pleural cavities next, and the pericardial cavity last. The cavities and their contents are to be inspected in the order and at the time that each is opened, but the organs are to

be removed from the cavities for further examination in the reverse order, beginning with the heart.

The *primary or long anterior incision to bare the thorax and to open the abdomen* (Fig. 5) should extend from the larynx to the pubes, passing to the left of the umbilicus, so as not to cut the round ligament. In cutting, the handle of the knife is depressed so as to use the belly of the blade rather

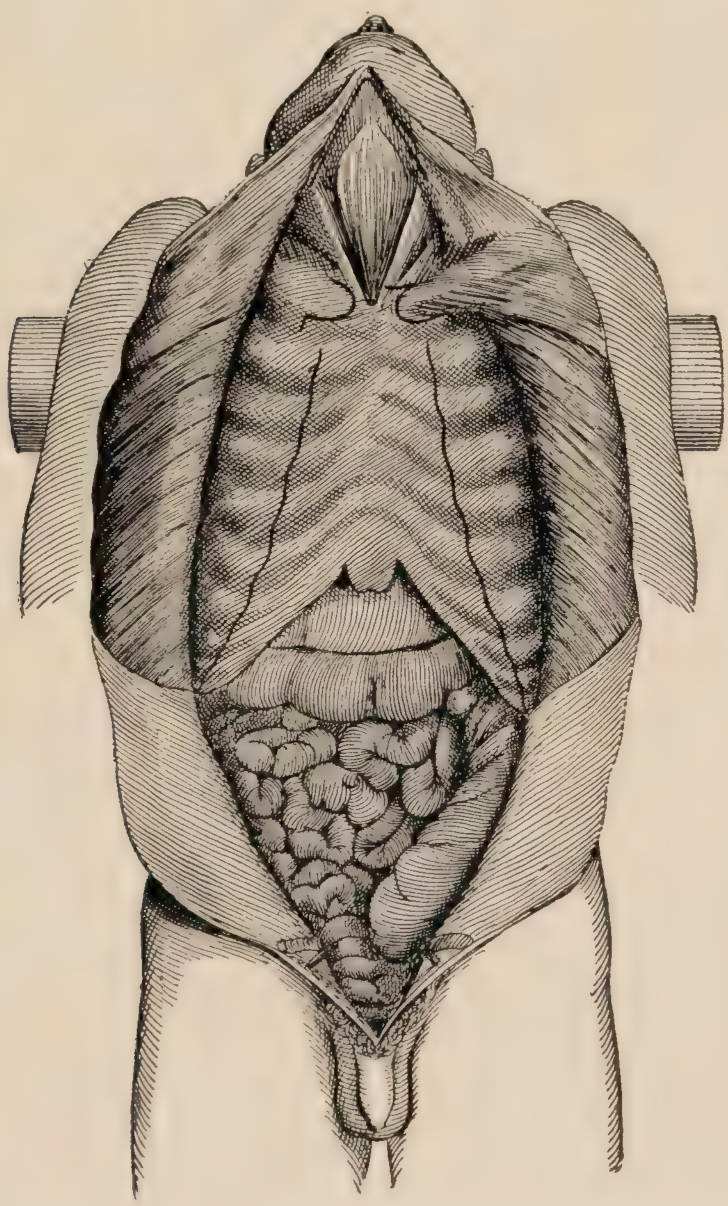

FIG. 5.—Primary incision in the body (Nauwerck).

than the point. An incision beginning as high as the chin is, unfortunately, rarely allowable. Over the sternum the cut should extend down to the bone; over the abdomen, however, only into the muscles, or in fat people through the muscles into the subperitoneal fat-tissue. To open the abdominal cavity, nick carefully through the peritoneum just below the sternum, introduce the first and second fingers of

the left hand, and while making strong upward and outward traction on the right abdominal flap extend the incision to the pubes. Some operators prefer to separate the fingers and to cut between them.

The abdominal flaps are rendered much less tense by cutting the pyramidales and recti muscles from below just above the pubis. Care must be taken not to injure the overlying skin. The abdominal cavity can now be examined, but more room will be obtained if the skin and the underlying muscles be first stripped back from the thorax to about 5 cm. outside of the costochondral line.

The operation is most easily and neatly done by lifting the skin directly away from the chest-wall or turning it forcibly out with the left hand, and then cutting the tense tissue close to the cartilages and ribs with long sweeps of the knife held almost flat. The operation begins over the lower border of the ribs and extends upward. In dissecting off the skin and muscles from the left side the right hand works underneath the left. The mammæ can easily be incised from the under side of the flap, and if necessary the axillary lymph-nodes can be reached by dissecting the skin farther out, especially over the clavicle. Before beginning the inspection of the peritoneal cavity it is important to examine first the surface of the incision into the abdomen, noting the thickness and color of the fat-tissue and the condition of the muscles.

Inspection of the Abdominal Cavity.—The character of any fluid present should be determined and its amount measured or estimated. The simplest way to remove it is to dip it up with a small cup or dish and pour it into a glass graduate for inspection and measurement. If the presence of gas within the peritoneal cavity is suspected, a small pouch should be formed in the first incision as soon as it has been made and water poured in. The first opening into the abdominal cavity should then be made with the point of a scalpel at the bottom of the water, through which the gas, if present, will escape in bubbles.

The various abdominal organs and their relations to each other are to be investigated *in situ* by sight and by touch.

As a rule, examine first the gastro-intestinal tract, including the appendix and the mesenteric lymph-nodes. Ulcerations of the intestine can often readily be made out through the walls. The examination of the spleen, liver, kidneys, and pelvic organs follows. The pancreas is easily reached by tearing through the omentum between the stomach and the colon, so as to open the lesser peritoneal cavity.

After the inspection of the abdominal organs the position of the diaphragm is to be ascertained on both sides in the costochondral line by measuring with the right hand passed palm upward underneath the ribs, and the left hand outside at the corresponding height to mark the position of ribs or intercostal spaces. On the right side the hand is to be passed up on the outside of the falciform ligament. Normally, the diaphragm stands at the fifth rib on the left side, and at the fourth rib or fourth interspace on the right.

Opening of the Thorax.—To open the thorax, cut through the cartilages close to the ribs from the second down (about 5 mm. distant) with a scalpel held nearly horizontal, so that as one cartilage is cut through the handle of the scalpel will strike the next below and prevent the blade from penetrating too far and injuring the lung. In young people the cartilages can be cut easily by one long stroke on each side, but care must be taken not to go too deep. If the intercostal muscles are not divided by the same operation, the sternum can be depressed by the left hand and the muscles severed by one pass of the knife on each side. The lower end of the sternum can now be elevated and freed from below upward from the diaphragm and pericardium until the first rib is reached. The cartilage of this rib is to be cut about 1 cm. farther out than the others, and from below upward toward the clavicle, with the handle of the knife beneath the elevated sternum and with the point and edge of the knife directed upward and a little outward. The sternum is then to be still further freed from the anterior mediastinal tissue until its upper end is reached. The sterno-clavicular joint on the left side can now be easily opened from below by entering a scalpel just above the cartilage of

the first rib, and following the irregular line of the joint around the end of the clavicle, while at the same time drawing the sternum over to the right side of the body. The right sterno-clavicular articulation is to be opened by continuing the incision of the scalpel over the upper end of the sternum and into the second joint. The advantage of this method is that there is much less danger of wounding the large vessels at the base of the neck, and thus of mingling blood with any exudation which may happen to be present in the pleural cavities. If preferred, however, the articulations can be opened and the cartilages of the first ribs cut from above before freeing the sternum from the diaphragm. In this case enter a short, sharp, narrow-bladed scalpel held vertically, but loosely, into the left joint on its upper side, starting the incision just outside of the attachment of the sternal end of the sterno-mastoid muscle, and cut around the end of the clavicle by a series of short up-and-down strokes, allowing the blade to follow the irregular line of the joint. After cutting through the joint continue the incision outward and cut through the cartilage of the first rib.

If the cartilages are calcified, use the costotome and cut through the ribs, as more room can be gained in this way, and they are more easily cut than calcified cartilages. When for any reason it is not permitted to open the thorax, the organs within it can be obtained through the opening into the abdominal cavity by freeing the diaphragm from the ribs, and removing first the heart and then the lungs. The sternum should be inspected at the time of its removal. It is perhaps best to examine next, especially in children, the epiphyses of the ribs at the costochondral line for any evidence of thickening.

Inspection of the Pleural Cavities.—In the pleural cavities, as in the peritoneal cavity, the character and amount of any abnormal contents must be determined. If, from the clinical history or from any other reason, the presence of air in a pleural cavity is suspected, a pouch should be formed over the ribs by aid of the skin-flap and filled with water. The pleural cavity is then to be pierced with a scalpel

through the bottom of the pouch. Air, if present, will bubble up through the water.

Slight adhesions are best torn through or cut. If the lungs are firmly attached, it is best to strip off the costal layer of the pleura with the lung. This is most easily done by starting the anterior edge of the costal pleura with the handle of the scalpel, and working in first a finger and then the whole hand until the pleura is entirely free. In passing the hand into the pleural cavities protect the back of it, especially if the ribs have been cut through, by folding the skin-flap in over the edge of the ribs.

If desired, the lungs can be drawn forward, examined over their whole extent, even incised, and then replaced until the heart has been removed. In the connective tissue of the *anterior mediastinum* there is almost always a certain amount of emphysema due to the removal of the sternum. Emphysema due to laceration of lung-tissue is more marked in the upper half of the mediastinum, and usually extends up into the neck. The thymus gland attains its full development at the end of the second year, after which time it usually gradually disappears.

Opening of the Pericardium.—To open the pericardium, seize the sac near the middle with fingers or forceps, snip through the wall with knife or scissors, and with either instrument cut upward to where the pericardium is reflected over the large vessels, downward to the lower right border, and lastly to the apex. By gently raising the apex of the heart the amount of fluid in the pericardial cavity can be seen. The normal amount is about a teaspoonful, but it may be increased to 100 c.c. in cases where the death-agony is prolonged. Pericardial adhesions should be broken through with the fingers. If this is impossible, the heart must be incised through the pericardium.

External Inspection of the Heart.—Determine first the position, size, and shape of the heart, and the degree of distention of the different parts. The right ventricle and both auricles are usually distended with blood, which may be fluid as in death from suffocation or more or less coagu-

lated. The left ventricle is contracted and empty unless the individual has died from paralysis of this part of the heart, when it will be found distended with blood (condition of greatest diastole).

Opening of the Heart.—The heart may be opened *in situ* or after removal from the body. Except in certain cases, to be spoken of later, it usually will be found advisable to remove the heart before making any incision into it, for the reason that it can be more perfectly opened after removal, especially by beginners, and the danger of contaminating any bacterial lesions of the valves is lessened.

To *remove the heart*, grasp it gently near the apex with the left hand, supporting it further, if necessary, by one or two fingers placed above the coronal suture, and lift the whole heart vertically upward. Then cut its vessels from below upward with the knife held transverse and oblique. Divide in turn the inferior vena cava, the pulmonary veins on both sides, the superior vena cava, the pulmonary artery, and the aorta. Go deep enough to remove the auricles entire, but avoid injury to the underlying esophagus.

For making the incisions to *open the heart* either a long, slender-bladed knife or long, straight scissors may be used. The heart should be placed on a board with its anterior surface up. The right auricle is opened by cutting from the orifice of the inferior vena cava into that of the superior, and from the latter into the auricular appendage. The first incision to open the right ventricle is made through the tricuspid valve and the wall of the ventricle along the under surface of the right border of the heart. It should be carried to the end of the ventricle, which does not reach quite to the apex of the heart. The second incision begins about the middle of the first, just above the insertion of the anterior papillary muscle (which should not be cut), and is carried through the pulmonary valve well over on the left side along the left border of a narrow, projecting ridge of fat-tissue usually present, so as to pass between the left anterior and the posterior segments of the valve.

The left auricle is opened in a manner similar to the right

by incisions joining the four orifices of the pulmonary veins and extending into the auricular appendage.

The first incision into the left ventricle is through the mitral valve along the left border of the heart (*i. e.* the middle of the external wall of the left ventricle), between the two bundles of papillary muscles, to the apex of the heart. The second incision begins at the termination of the first at the apex, and is carried up close to the interventricular septum, parallel to the descending branch of the

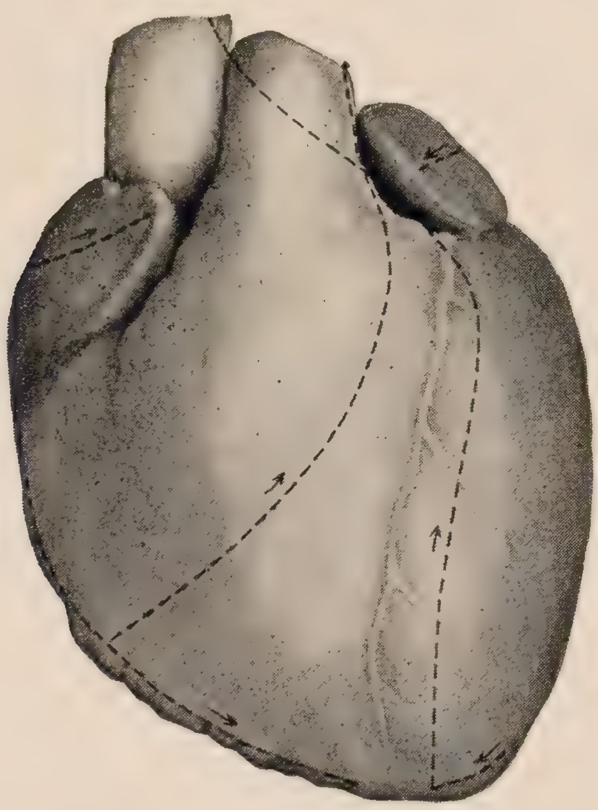

FIG. 6.—Heart, showing incisions.

anterior coronary artery and about 1 cm. from it. The upper portion of the incision should pass midway between the pulmonary valve and the left auricular appendage. Ordinarily, one of the aortic cusps is divided, but this may be avoided, if desired, by dissecting away to some extent the pulmonary artery from the aorta and carrying the incision well over to the right between the right posterior and anterior valve-segments. As each auricle is opened the blood and clots it contains should be carefully removed and the auriculo-ventricular valves carefully inspected from above. In certain cases—as, for instance, extreme stenosis—it may be preferable not to cut through the valve, but to begin the

incision in the ventricular wall below the valve. The ventricular cavities should in like manner be freed from clots and the valves closely inspected. The coronary arteries should always be opened by means of small, narrow-bladed, probe-pointed scissors as far as they can be followed. The examination of the descending branch of the anterior artery is especially important. The posterior coronary is best opened by placing the tip of the left fore finger in the aorta over the orifice of the artery, and cutting from without in toward the finger-tip until the vessel is reached, when it can easily be slit up. In this way injury to the aorta is avoided.

In cases of more or less sudden death with symptoms of asphyxia the pulmonary artery should always be opened *in situ* before removal of the heart, in order to examine for possible emboli, because they often lodge just at the point where the vessels are severed in removing the heart and lungs, and easily may slip out unobserved. The simplest operation is to thrust a sharp-pointed scalpel through the artery just above the valve on the left side in the line of incision already described, and to cut upward until the branches to the right and left lungs are reached. If desired, this incision may be extended down through the pulmonary valve and the ventricular wall along the line given for the second incision in the right ventricle.

The water-test for the competence of the valves of the heart is not very reliable, especially for the auriculo-ventricular valves, and is not so much used as formerly. Inspection and measurement of the valve after the heart has been opened will usually enable one to judge fairly accurately concerning the degree of competence. Before applying the test to the aortic valve the first incision into the left ventricle must be made and the cavity freed from clots, so that no obstruction will exist below the valve. Then the heart is to be held so that the aortic valve is perfectly horizontal, and water poured in from above to float the cusps out. If competent, they should keep the water from flowing through. If, however, in holding the heart the normal relations of the valve and the surrounding parts are not

maintained, the valve may leak. A second source of error is that the water may escape through the coronary arteries, branches of which have been cut in opening the ventricle. In testing the mitral valve the left auricle is first opened and the clots removed, so as to expose the upper surface of the valve. Then the nozzle of a syringe is introduced through the aortic valve and water forced in so as to float the mitral curtains up. The test, however, is very unreliable, because the parts cannot be placed under natural conditions.

The pulmonary and tricuspid valves can, of course, be tested by methods similar to those already described.

Increase or diminution in the size of the heart is best determined by weighing the organ after the removal of the clots. In certain cases, however, and in special investigations measurements of different parts of the heart are desirable. Roughly, the heart is the size of the individual's fist.

The following *weights* and *measurements* are taken from *Nauwerck's Sectionstechnik:*

Weight of the heart averages in men,	300 gr.	*Orth.*
“ “ “ “ women,	250 “	
Krause gives the average weight of the heart as 292 gr.		
Relative weight of heart to body in men,	1–169	*Krause.*
“ “ “ “ women,	1–162	
Length of heart in men,	8.5–9 cm.	*Bizot.*
“ “ women,	8.0–8.5 “	
Circumference of heart at base of ventricles,	28.8 cm.	(*Sappey*).
Thickness of wall of left ventricle,	1.1–1.4 cm.	*Krause.*
“ “ right “	0.5–0.7 “	
Thickness of wall of left ventricle (without trabeculæ),	7–10 mm.	*Orth.*
“ “ right “ “ “	2–3 “	
Circumference of mitral orifice,	10.4 (W.), 10.9 (M.)	*Krause.*
“ “ tricuspid “	12.0 (W.), 12.7 (M.)	
“ “ aortic “	7.7 (W.), 8.0 (M.)	
“ “ pulmonary orifice,	8.9 (W.), 9.2 (M.)	
“ “ ascending aorta,	7.4 cm.	
“ “ pulmonary artery,	8.0 cm.	(*Buhl*).

The directions given for the removal and opening of the heart apply only when the organ is normal or contains lesions within itself which are not in continuity with any of the vessels entering into it. In aneurysm of the ascending

aorta, in thrombosis of a vena cava, and in a number of different lesions connected with the heart or with the vessels given off from it, it is important to examine these vessels and to open them while they are still in continuity with the heart. For this purpose it is often necessary or advantageous to remove the thoracic organs in one piece, so as to be able to examine the central circulatory apparatus in continuity from the front and back before disturbing any of its relations. This is done by cutting across the trachea and adjoining tissues as high in the neck as necessary or possible, and dissecting them free from the cervical vertebræ and the first ribs. Then by drawing the trachea and surrounding tissues forcibly forward the aorta and overlying organs can be easily stripped from the vertebral column as low as the diaphragm. The left hand is now placed around the lower end of the pericardial sac, the aorta, and the esophagus just above the diaphragm, and the vessels are severed by cutting between the hand and the diaphragm.

More space for the examination *in situ* of the vessels at the base of the neck can be obtained by freeing the clavicles from all attachments above and to the first ribs and drawing them forcibly outward; this operation will be found especially useful in following up the subclavian vessels.

Removal of the Lungs.—Pleural adhesions have already been spoken of. If the base of the lung is adherent to the diaphragm, it is usually advisable to remove the latter with the lung by cutting through its insertion into the ribs. According to Orth, there is less danger of wounding the abdominal organs if scissors be used for the performance of the operation. After the lung is free it is drawn forward out of the pleural cavity, and the root of it is grasped from above downward between the separated fingers (first and second or second and third) of the left hand. The lung, thus resting in the palm of the left hand, is first drawn downward toward the pubes until the primary bronchus is divided by a nearly vertical incision above and behind the left hand. Then the lung is lifted vertically upward, and the rest of its attachments cut in the same direction from above

downward by the knife held transverse and flat, so as to avoid injuring the esophagus and aorta.

The procedure is the same for both lungs. Once in a great while the apex of a lung will be found so firmly adherent by dense scar-tissue that it can be freed only by using the knife.

The primary or main incision into a lung is a long, deep

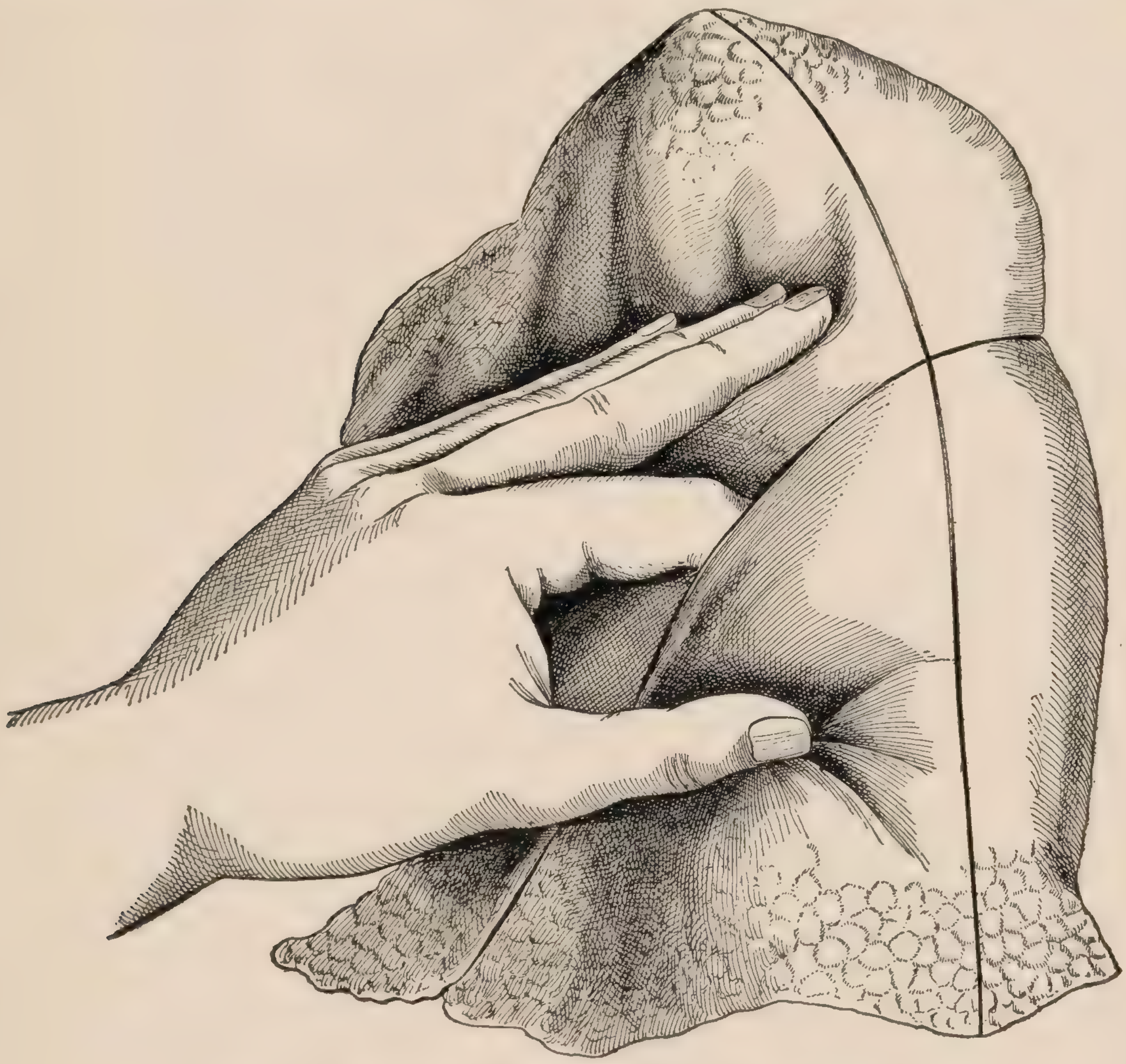

FIG. 7.—Method of incising the lung (Nauwerck).

cut from the apex to the base and from the convex surface to the root, slitting the primary bronchus, and thus not cutting it off from its branches to the upper and lower lobes (Fig. 7). To incise the left lung, place it with its inner or median surface and root downward on a board and with its base toward the operator. The left thumb steadies the lower lobe; the first finger reaches between the two lobes almost to the primary bronchus; and the rest of the fingers should hold the upper lobe.

The right lung is most easily incised by placing it in the same position, but with the apex toward the operator; in other words, always place the anterior edge of a lung beneath the palm of the hand. Some prefer to place each lung on its lower or diaphragmatic surface for incision. The right middle lobe is incised separately by a cut extending transversely in its greatest diameter.

The bronchi and blood-vessels should be opened up for some distance with small probe-pointed scissors—as a rule from the surface of the section—cutting through the overlying lung-tissue. In some cases, however, it is best to open up both the blood-vessels and the bronchi from the outside of the lung before incising it. The order to follow is vein first, then artery, and finally the bronchus.

Secondary cuts into the lung are to be made parallel to the main incision.

The bronchial lymph-nodes should be incised from the outside of the lung.

Organs of the Neck.—The operation of the removal of the organs of the neck is greatly facilitated if it is possible to continue the primary skin-incision up to the chin. In other cases dissect the skin from the larynx and muscles of the neck as far up as possible. In like manner free the muscles, esophagus, and trachea from their attachments laterally and posteriorly. Then allow the head to drop well back over the end of the table, and pass a long, slender-bladed knife up between the skin and the larynx, just behind the symphysis of the lower jaw, until the point of the knife appears beneath the tip of the tongue. From this point the knife is carried with a sawing motion down first one ramus of the jaw and then the other, dividing laterally the glossal muscles as far back as the posterior pharynx. The knife is next carried up behind the esophagus, and the posterior wall of the pharynx divided as high as possible. Pass the left hand up inside of the neck and draw down the tongue. Then cut the attachments of the soft to the hard palate, carrying the knife well out so as not to injure the tonsils. Any remaining attachments are usually easily severed by

pressing the tongue first to one side and then to the other, and cutting close to the roof of the pharynx.

Each lobe of the thyroid gland is to be incised in its greatest diameter.

Next cut through the middle of the uvula and examine all of the pharynx removed. Incise the tonsils vertically. The esophagus is to be slit in the median line posteriorly; if it is normal, the larynx and trachea are then slit in the posterior median line also, thus splitting the esophagus in two.

The Abdominal Cavity.—The order of removal of the abdominal organs varies with different operators, and under varying circumstances with the same operator. The gastro-intestinal tract, including the liver and pancreas, may be removed before or after the genito-urinary tract. The spleen as an organ by itself is often the first to be removed. The early removal of the liver is occasionally advantageous for the sake of the additional space obtained for the examination of the other organs. It is well to practise the different methods of procedure, so that in a difficult case the best may be selected, because the examination of the abdominal cavity, especially in cases of extensive disease with numerous adhesions, is often one of the hardest tasks in post-mortem technique. As a rule, it is best to follow the usual order as long as possible, gradually removing the more or less normal or uninvolved organs. Occasionally it may be advisable to remove the organs *en bloc*, so as to be able to approach the problem from all sides.

In all cases of acute peritonitis it is best before removing any organ to search for the source of the infection, paying particular attention to the vermiform appendix, to the gastro-intestinal tract, and, in females, to the pelvic organs.

The order of removal of the abdominal organs adopted in this book for the majority of cases is that which seems the simplest and most natural—namely, to remove first the spleen as an organ essentially by itself; secondly, the gastro-intestinal tract, including the pancreas and liver, which forms the upper layer; thirdly, the genito-urinary tract or middle layer, leaving the circulatory tract, the lowest layer, to be opened and inspected *in situ*. If, however, it proves neces-

sary to open a part of the gastro-intestinal tract *in situ*, it will be neater perhaps to remove the kidneys and spleen first. Occasionally at private autopsies it may be unnecessary to examine the intestinal tract; under these circumstances it is important to be able to get at the different organs without taking out the intestines.

The Spleen.—As a rule, the spleen can easily be drawn forward from its bed behind the fundus of the stomach, beneath the diaphragm, and lifted on to the lower edge of the ribs on the left side without cutting its vessels. The organ is then to be incised in its greatest diameter while thus firmly fixed between the left hand and the ribs; or the vessels may be cut close to the hilus and the spleen incised after being placed on a board.

In cases of adhesion to the diaphragm the spleen must be handled carefully while the fibrous attachments are torn or cut through, for the capsule is easily ruptured. Occasionally it is advisable to cut out with the spleen the portion of diaphragm attached to it.

The important anatomical structures to be noted in the macroscopic examination are the capsule, trabeculæ, blood-vessels, lymph-nodules, and pulp. The weight of the spleen, according to Orth, varies from 150 to 250 grams. The average weight is put at 171 grams. The spleen measures $12 \times 7.5 \times 3$ cm.

The Gastro-intestinal Tract.—The first step is to examine externally, more or less carefully according to the clinical symptoms, the whole tract from the stomach to the rectum, if it has not already been done at the primary inspection of the peritoneal cavity. The main points to notice are distention or contraction of the intestines, injection of the blood-vessels, thickening of the wall, especially in the lower part of the ileum, adhesions, exudations, etc. Inspect the mesentery, its length, the amount of fat, and the size of the lymph-nodes; incise the latter to determine color and consistency. Examine the mesenteric vessels if any evidence of infarction of the intestine is noticed. The portal vein and its branches should be opened up *in situ*, in all cases of ab-

scess of the liver or of secondary deposits in it of malignant growths, before the gastro-intestinal tract is removed. As a rule, it is not necessary to open any part of the gastro-intestinal tract *in situ.* The operation can be performed much more neatly at the sink. The duodenum is often opened for the sake of investigating the flow of bile from the gall-duct, but except in cases of jaundice the operation must be looked upon largely as a physiological experiment.

Free the *omentum* from the transverse colon by putting it on the stretch and dividing it with the knife close to the colon. Then begin the removal of the large intestine by drawing the sigmoid flexure forcibly forward and cutting the mesocolon close to the gut, first down to the rectum, then upward to the transverse colon. Free the latter by dividing the two folds of the lesser omentum, if not already cut through, which unite it to the stomach. The ascending colon is to be freed in the same manner as the descending portion. Care should be taken not to injure the appendix. If the lower part of the sigmoid flexure be now stripped upward a short distance with the fingers, so as to force the intestinal contents out of the way, the gut can be divided just above the rectum without fear of the feces escaping.

Place the freed intestine in a pan or pail, and as the small intestine is divided from its mesentery deposit it in the same receptacle. To remove the small intestine, begin at the cecum, and, while lifting the ileum with the left hand strongly enough to keep the mesentery constantly tense, cut the latter close to the intestine by playing the knife easily backward and forward across it with a fiddle-bow movement. Continue the operation until the duodenum is reached. The mesentery can now be dissected from the duodenum and removed, or the mesentery, duodenum, pancreas, and stomach can be removed in continuity with the intestine by carefully dissecting them off the underlying structures. The operation is perhaps more easily accomplished by freeing the organs from below upward. First cut down through the diaphragm and free it around the esophagus. Then separate the stomach from the liver by means of the thumb and fingers

of the left hand in such a way as to put on the stretch the vessels of the hepato-duodenal ligament. These vessels (hepatic artery, common gall-duct, and portal vein) are then carefully divided in the order named. As each vessel is cut the character of its contents should be observed to see if anything abnormal is present.

The mesentery, if still present, the duodenum, the pancreas, and the stomach, are now to be dissected carefully away from the underlying vessels from below upward until the esophagus is reached. This may be constricted by the fingers at any point desired, and cut across without danger of the gastric contents escaping and without the necessity of tying. In certain cases of hemorrhage from the stomach associated with cirrhosis of the liver it is important to remove the esophagus in continuity with the stomach, because in these cases the hemorrhage usually takes place from dilated esophageal veins.

The *stomach* and *intestines* are now to be opened at the sink by means of the enterotome, the colon along one of its longitudinal muscular bands, the small intestine along its mesenteric attachment, because the most important lesions usually occur opposite this line in the lymph-nodules and Peyer's patches. The stomach is opened by many along the greater curvature; others, however, prefer to cut along a line 3 cm. from the lesser curvature, on the ground that better museum preparations are thus obtained. In case any tumor or focal lesion is perceived from the outside, it is advisable to cut the stomach, if possible, in such a way as to leave the pathological part uninjured.

Whenever jaundice is present the duodenum must be opened *in situ* in order to examine the bile apparatus in continuity, so as to determine whether the coloring is due to obstruction of the hepatic or common gall-ducts, or is of so-called hematogenous origin.

To open the *duodenum* make a transverse fold in the anterior wall and incise with the scissors. Continue the longitudinal slit thus made up as far as the pylorus and down to where the duodenum passes beneath the mesentery. Notice

the contents of the duodenum and their color both above and below the opening of the gall-duct. The ductus choledochus usually opens in common with the ductus pancreaticus on the posterior wall of the duodenum a little below the middle of the head of the pancreas, at a point marked by a small papilla which can easily be recognized by putting the mucous membrane on the stretch transversely. Press first on the common duct gently and in the direction of the papilla, watching the opening to see if any obstructing material is forced out. Pressure is then to be made on the gall-bladder to see if its contents also will flow. If necessary, the common duct and its branches are to be opened *in situ*. In certain cases the ductus pancreaticus is likewise to be opened up.

Several cross-sections of the *pancreas* are usually better than one in the greatest diameter, because the duct is left in a better condition for slitting up if necessary. The weight of the pancreas varies from 90 to 120 grams (Orth). It measures $23 \times 4.5 \times 2.8$ cm.

The Liver.—The liver is usually the last organ of the gastro-intestinal tract to be removed. This is ordinarily done by lifting up the right lobe and freeing it from all attachments as far as the vertebral column: the right lobe is then lifted and placed on the edge of the ribs on the right side, while the left lobe is elevated and freed. If the diaphragm is firmly adherent, remove it with the liver. The incision to display the liver is a long deep cut passing through the right and left lobes in the greatest diameter of the organ.

In a good many cases it is very convenient to remove the liver at the beginning of the special examination of the abdominal cavity, because more room can be obtained for the investigation of the other organs. This latter fault can to some extent be obviated by cutting the diaphragm on the right side and allowing the liver to slide forward somewhat into the right thoracic cavity.

There can be no objection to the removal of the liver when jaundice is not present or when the liver is not connected by continuity with the lesion of some other organ

(pylephlebitis, malignant growth extending through portal vein or along gall-ducts, etc.).

The operation is performed as follows: Pass the left hand in between the diaphragm and the right lobe and push the liver forward out of the right hypochondrium. Incise it deeply in its greatest diameter through the left and right lobes. Next free the gall-bladder from its bed by means of the fingers, and cut it off near the ductus hepaticus after compressing its lower end. It can then be opened lengthwise and washed without danger of discoloring the liver or other organs. The liver is now to be grasped by placing the thumb on the under surface of the liver and the fingers in the incision. Elevate the organ, and, while carefully watching, cut through the hepato-duodenal ligament, which includes the blood-vessels and the ductus hepaticus. The ligamentum hepato-gastrium, the inferior vena cava, the suspensory ligament, the ligamentum coronarium, and the tissue between the inferior surface of the liver and the upper end of the kidney follow next: the adrenal is to be left on the kidney, and the diaphragm ought not to be injured.

Even in the ordinary way of removing the liver the organ will be found much easier to handle if the usual incision is made *in situ*, so as to furnish a hold for the left hand.

Other cuts into the liver are best made parallel to the primary one.

Orth gives the weight of the liver for adults as varying from 1000 to 2000 grams. The average weight is usually put at 1500 to 1800 grams.

The liver measurements are as follows:

Length from right to left	25–32	cm.
Width of right lobe	18–20	"
Width of left lobe	8–10	"
Vertical diameter of right lobe	20–22	"
Vertical diameter of left lobe	15–16	"
Greatest thickness	6– 9.5	"

The Kidneys and Adrenals.—If the adrenals are to be removed with the kidneys, it is necessary to cut first to the inside, and secondly above the adrenal, and then to make

from the outer end of the second cut a curved incision along the outer convex border of the kidney through the peritoneum and the perinephritic fat-tissue. The left hand is to be inserted into the cut, the mass of tissue drawn forcibly forward, and the vessels divided as close to the aorta as possible, so that the renal vessels may be slit up and examined in connection with the renal lesions. The adrenal should be incised crosswise. The kidney is to be held firmly in the left hand between the thumb and fingers while a longitudinal incision is made from the convex border to the hilus. As a rule, it is better to shell it out of its investing fat-tissue before incising it.

It will often be found convenient to make simply the curved incision above given, to shell the kidney out of its fat-capsule, and then to divide its vessels, leaving the adrenal behind to be incised *in situ* or removed separately. As a rule the left kidney is removed first.

In all cases in which the bladder is involved in pathological changes in common with the kidneys the whole urinary tract should be removed intact, so that the lesions may be examined in continuity. For this reason it is a good plan to open up the pelvis of the kidney and the ureter from the primary incision, in order to see if any lesion is present before dividing the ureter.

If it is desired to remove the kidneys before the intestines, the latter must to some extent be freed from their normal attachments.

The splenic flexure of the colon is first to be drawn forcibly forward and its attachments divided where they hide the left kidney. If the ureter is to be taken out also, it is best to free the whole of the descending colon from its mesocolon. Then the colon and the coils of small intestine are drawn over to the right side of the body, so as to leave the left kidney and adrenal exposed. They are then removed in exactly the same manner as already described.

To remove the right kidney the hepatic flexure must be freed from over it. If the ureter is to be taken out, the descending colon and the cecum are dissected from over it.

The right adrenal is firmly attached to the under surface of the liver, and must be carefully dissected from it by turning the latter upward.

If the urinary tract is to be removed in continuity, each ureter is dissected down to the brim of the pelvis, and then left with its kidney attached until the pelvic organs have been taken out.

After the kidney has been incised the capsule is to be stripped off, at least in part, so that the appearance of the surface of the kidney and the presence or absence of adhesions between the capsule and the renal tissue can be determined.

The points to be noted in the macroscopic examination of the kidney are size, consistency, and, on section, color, relative proportion of cortex to pyramids, and thickness of each; finally, the normal markings of the kidney, including blood-vessels, glomeruli, convoluted and straight tubules of cortex, collecting tubules of pyramids.

The average weight of the kidney is 150 grams. The left kidney is always 5 to 7 grams heavier than the right (Orth). A kidney measures $11–12 \times 5–6 \times 3–4.5$ cm. The cortex measures in thickness 4–6 mm. The relation of the cortex to the medulla is 1 to 3.

The Pelvic Organs.—The pelvic organs are most easily and neatly removed by stripping the peritoneum from the pelvic wall with the fingers. Begin over the bladder and extend down the sides of the pelvis until the fingers meet beneath the rectum. Brace the backs of the hands laterally on the brim of the pelvis and lift the fingers forcibly upward; this movement will free the pelvic organs cleanly from the sacrum, and leave them attached only anteriorly at the rectal and genital openings, and posteriorly by the peritoneum and the vessels at the brim of the pelvis.

Anteriorly, the attachments may now be divided with the knife at whatever point seems advisable, ordinarily close to the pubes just anterior to the prostate (or through the urethra and vagina in females) and through the lower end of the rectum. Posteriorly, cut through the tissues at the brim

of the pelvis, taking care not to cut the ureters if the kidneys are still attached to them. The *rectum* is to be opened with the enterotome along the posterior wall, and the inner surface thoroughly washed off so as to avoid soiling the other organs.

To open the *bladder* in males, especially if the penis has been removed in continuity with it, incise with the scissors a transverse fold in the anterior wall of the fundus, and carry the incision through the urethra and along the dorsum of the penis. To accomplish the latter act perfectly the *penis* must be firmly stretched by having an assistant pull at the frenum while the bladder is held fixed by the operator.

In females it is usual to enter the scissors into the bladder through the urethra and to cut through the middle of the anterior wall of the fundus.

In males the *rectum* should be dissected from the bladder, so as to lay bare the vesiculæ seminales and the prostate, which are examined by means of several transverse incisions.

In females, if the bladder is normal, the *vagina* is incised in the anterior wall through the middle of the bladder. Or the vagina may be incised laterally until the cervix is reached, and then the cut be carried up to the median line.

The *uterus* is incised in its anterior wall from the cervix to the fundus. From the upper part of this incision secondary incisions are carried out on each side to the orifices of the Fallopian tubes.

The *ovaries* are incised in their greatest diameter, from the convex border to the hilus. Weight of ovaries, 7 grams.

The *testicles* can readily be examined without external injury to the scrotum by cutting underneath the skin over the pubes down to the scrotum on either side of the penis, and shoving the testicles up through the incision. Cut carefully through the overlying tissues until the cavity of the tunica vaginalis is opened. Remove the testicle by severing the cord. The incision to display a testicle should be in the long diameter, beginning on the side opposite the epididymis and extending through into it. Weight of testicles, 15–24.5 grams. In cases of tuberculosis of the testis and epididymis

it is advisable not to cut through the cord, but to remove the testicles and cords with the bladder, so that the whole genital tract may be examined in continuity and the associated lesions in the vesiculæ seminales demonstrated, if present.

The *penis*, or at least the larger portion of it, can be removed in connection with the bladder by continuing the primary body-incision out to about the middle of the dorsum of the penis, which is then to be freed from the investing skin and divided just posterior to the corona. It is next dissected back to the pubic arch, and freed from it partly by cutting from without, partly from within, the pelvis, until the penis can be passed underneath the arch into the pelvis. Other methods are to cut through the symphysis, which can then readily be sprung apart by swinging one of the legs out in a horizontal plane, or even to saw out a small section of bone including the symphysis, so as to have more room for freeing the attachment of the penis and for removing it.

The structures now remaining in the abdominal and thoracic cavities which require examination are the large blood-vessels, the thoracic duct, the celiac ganglion, and the retroperitoneal lymph-nodes. The *inferior vena cava* and its branches are first examined (especially in all cases of pulmonary embolism) by slitting them with scissors along the anterior wall. If it is necessary to follow the iliac vessels into the thigh, it will be found easier in sewing up if the primary abdominal incision is continued off to the side in question, thus giving a single though curved incision.

It is sometimes advisable to open up the inferior vena cava and its branches before removing the pelvic organs, so that thrombi extending into the pelvic vessels may be examined before they are disturbed.

The *semilunar ganglia* lie on the aorta, around the celiac axis, above the pancreas.

The *thoracic duct* lies behind and to the right of the aorta. In the thorax it is most easily found by dissecting on the right side between the aorta and the azygos vein. The re-

ceptaculum chyli lies to the right and behind the aorta upon the second or third lumbar vertebra. Examination of the thoracic duct is of especial importance in cases of tuberculosis of the intestine and mesenteric lymph-nodes with secondary miliary tuberculosis.

The *aorta* is to be opened *in situ* along the anterior wall throughout its whole extent, and the iliacs as far as the femoral ring.

Besides the brain, the spinal cord, and the thoracic and abdominal organs, it is often necessary to examine or remove for study other portions of the body that are affected by disease. A little ingenuity will enable one in appropriate cases to get at almost any part desired.

A view of the marrow in a long bone is most easily obtained in the femur by extending the body-incision down over one of the thighs, dissecting the muscles way, and then chiselling off a portion of the upper part of the shaft.

In tuberculosis of the spine it is quite easy to remove any part, or even the whole, of the vertebral column, including the pelvis and portions of the femurs, without other incisions than the one from the neck to the pubes, with extension down the thighs in case parts of the femurs are to be taken out. Divide the ribs a few centimeters from the vertebral column on each side of the portion that is to be removed, cut through intervertebral disks both above and below it, and then carefully dissect it free, taking great care not to button-hole the skin.

Removal of the Brain.—The incision into the scalp should begin from one to two centimeters behind the right ear, near its lower border, at the edge of the hair, and extend over the vertex of the skull to a corresponding point behind the left ear. The cut is most easily made by thrusting a small narrow-bladed scalpel, with its back toward the calvarium and its point toward the vertex, through the skin behind the ear and shoving it along in the desired direction. By making the incision in this manner the hair is not cut, but simply parted. The anterior flap should be stripped from the calvarium and the temporal muscles by putting it on

the stretch and dividing the loose connective tissue holding it by sweeping strokes of the scalpel nearly as far forward as the orbits. After a part of the flap has been freed it is often possible to strip the rest without using the scalpel. For the posterior flap, which should be removed back as far as the occipital protuberance, the scalpel nearly always has to be used.

If the hair is long, the anterior portion can be rolled into the anterior flap over the face and thus protected. The posterior portion is gathered at the nape of the neck, and then a towel is wrapped tightly around the head and neck, extending from the line where the flaps are reflected down to the shoulders, and is pinned over the lower part of the forehead. In this manner the hair is perfectly protected from being soiled and ample room is left for work.

Of the two methods of opening the skull, the circular and the wedge-shaped, the former makes the better museum preparation, but the latter is in greater use in this country, and has the advantage of rendering the calvarium less likely to slip out of place after the head has been sewed up.

The wedge-shaped incision consists of three cuts, which should be outlined on the periosteum of the skull with a scalpel. The first cut begins just above and behind the left ear, and is carried over the forehead just back of the edge of the hair or over the frontal eminences to a corresponding point above and behind the right ear. The two other cuts begin at each end of the first incision, forming there an obtuse angle, and are carried back to meet in the median line behind at an angle of about 160° a little in front of the occipital protuberance. The temporal muscle on each side is now to be scraped back from the line of incision out of the way of the saw, but is not to be cut off. The holder, if one is used, is attached with a foot in each obtuse angle in the temporal region. If a holder is not employed, the head is best steadied by hands on the calvarium and face. Use towels or cloth to prevent slipping.

Start the incision with the saw over the forehead and extend it back along the line marked out. It is best not to

carry the incision clear through the inner table of the bone, for two reasons: first, on account of the danger of injuring the brain-substance; secondly, because if the inner table or a part of it is cracked through with a chisel and hammer, it can be done without injuring the underlying tissue, and the irregular overlapping fragments of bone thereby formed serve afterward for holding the calvarium firmly and steadily in place.

After sawing along the lines marked out, insert a chisel in the frontal region, and with a quick, sharp blow crack through the rest of the inner table. In like manner insert the chisel in the middle of the other incisions and free the calvarium posteriorly. To remove the calvarium insert the chisel end of the hammer in the incision in the frontal region, and press down with the left hand while swinging the handle around in a horizontal plane.

By means of the powerful purchase obtained the calvarium is easily started. Then catch the hook of the hammer over the calvarium and strip it off. If the dura is adherent to the calvarium, it may be freed by using the point of the closed enterotome to pry it off.

In young children, and sometimes in old people, it is necessary to remove the dura with the calvarium. To do this, cut through the dura with the point of a scalpel along the lines of incision in the skull; then cut the falx cerebri in the median line, both anteriorly and posteriorly.

An infant's skull is best opened by cutting with a pair of scissors through the dura along the sutures (in the longitudinal suture on each side of the falx) well down to the floor of the skull. This gives five bone-flaps which may be turned out like the petals of a flower, leaving the brain uninjured. It is often necessary to cut half of the base of each flap in a horizontal line to aid its being turned out. The falx cerebri must of course be divided anteriorly and drawn back before the brain is removed. In sewing up, the bone-flaps are turned in over a bag of sand or sawdust filling the cranial cavity, and are kept perfectly in place by the skin.

In a case of fracture of the skull no cracking with hammer

and chisel is allowable; the calvarium must be freed entirely by sawing. The calvarium should be examined at the time of removal.

The next step is to inspect the dura. Under normal conditions it is not tense in the frontal region, but can be picked up with the forceps or fingers. If the dura is not thickened, the convolutions normally should be visible through it. The longitudinal sinus is opened with knife or scissors and its contents examined. Pacchionian granulations are not infrequently found projecting into it.

To remove the dura, cut through it with scissors or knife along the same lines in which the calvarium was sawn. Turn back each half of the dura and examine the surface of the convolutions and the inner surface of the dura. The convolutions should be distinct and rounded, not flattened, with obliteration of the gyri, as occurs when there is internal pressure.

The Pacchionian granulations are situated along the longitudinal fissure and may grow through the dura and form depressions in the calvarium. There may be apparent adhesions between the dura and pia due to veins passing from one to the other. The dura is still further freed by seizing the two halves anteriorly and lifting them up until the falx is tense at its insertion into the crista galli. Pass a knife in parallel to the falx, on the left side, with the edge forward, as far as the cribriform plate; turn it to the right and cut until the falx yields. Withdraw the knife in the same manner in which it was inserted. Next draw the dura back. It is usually more or less attached along the longitudinal fissure by Pacchionian granulations and by blood-vessels. These may be cut or torn through. Do not cut the dura posteriorly, but let it hang down.

To remove the brain, insert the two fore fingers, or the first and second fingers of the left hand, anteriorly between the dura and the frontal lobes, one on each side of the falx cerebri, and draw the brain gently back until the optic nerves are visible. Ordinarily, the olfactory nerves come away from the cribriform plate without trouble, but sometimes have to

be freed with the point of the knife. With a long, slender-bladed knife divide the optic nerves as far forward as possible while holding the brain back with the left hand. Continue to draw the brain carefully back and divide the cranial nerves

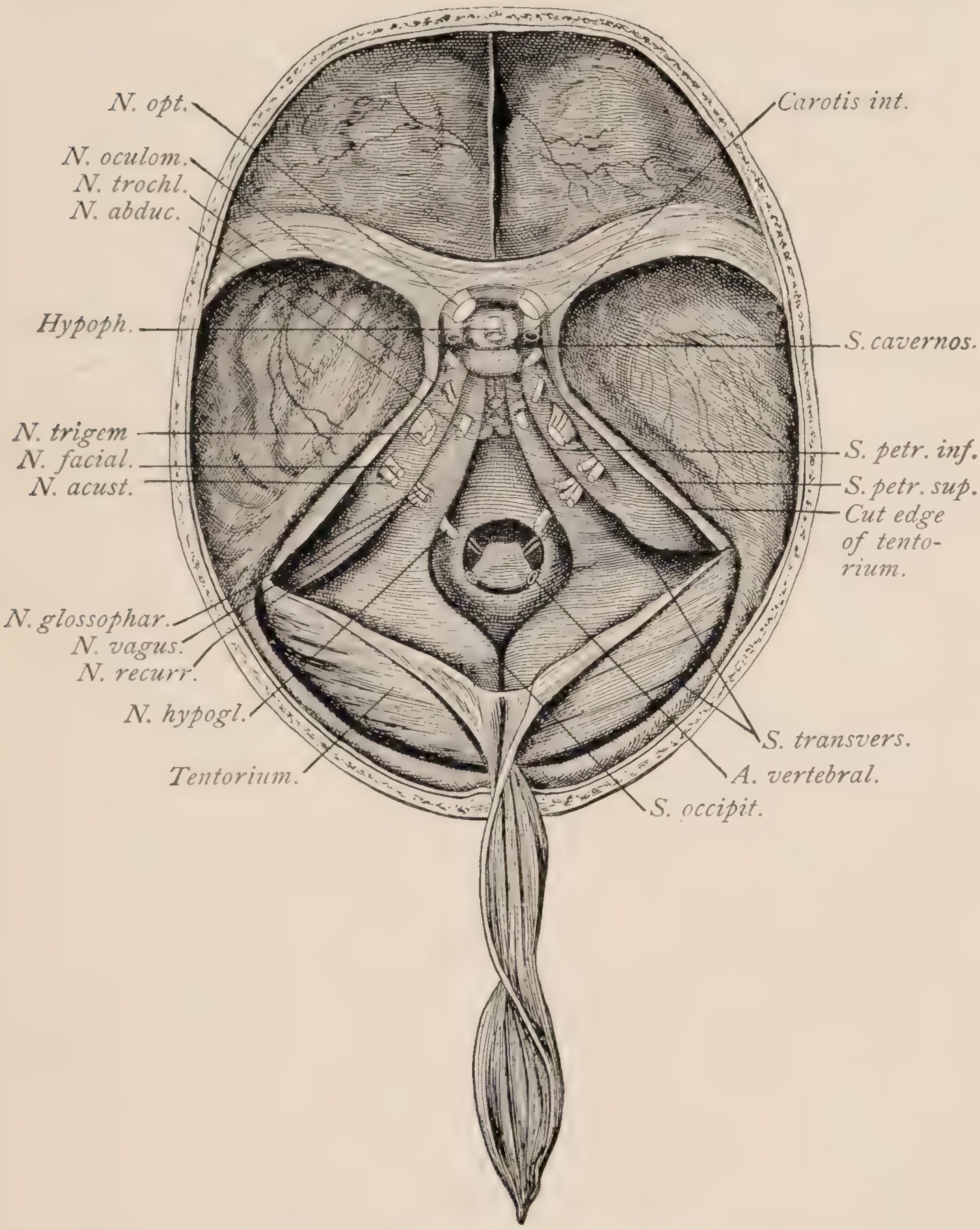

FIG. 8.—Base of skull (Nauwerck).

and the carotids. Then draw forward first the left, then the right temporal lobe, and cut the tentorium close to its attachment to the petrous portion of the temporal bone with a sawing motion, using the tip of the knife. Insert the knife at the side close to the squamous bone, and cut from there

in toward the foramen magnum. Then cut the nerves given off from the medulla oblongata while supporting the convexity of the brain in the left hand.

Lastly, carry the knife as far as possible into the spinal canal, and divide the cervical cord by an oblique incision from each side, severing the vertebral arteries with the same stroke. Better than a knife is the myelotome, because it gives a cross-section of the cord and allows more of it to be removed.

The brain is now to be removed by passing the first and second fingers of the right hand in on either side of the cord, and everting the brain while still supporting it posteriorly with the left hand.

Before proceeding to open the brain it is best to examine the base of the skull, particularly the dura, of which the sinuses should be incised, and the hypophysis cerebri.

If there is a suspicion of a fracture at the base, strip off the dura, so as to give a better opportunity for examination of the bone.

The brain should be weighed before it is dissected. The average weight in an adult male is 1358 grams; in an adult woman, 1235 grams.

External Examination of the Brain.—Place the brain with the base uppermost and with the cerebellum toward the operator. Examine first the pia and the cranial nerves, then the arteries, especially the middle cerebral and its branches on each side in the fissure of Sylvius, for it is here that emboli most frequently lodge. The pia bridging the fissure of Sylvius can sometimes be torn through, but usually has to be cut.

It is important, particularly in cases of obscure cerebral symptoms, to feel gently with the finger-tips all over the surface of the brain for any areas of increased density, because patches of sclerosis may in that way be found which might otherwise be overlooked.

By stripping off the pia—a procedure not often advisable—adhesions over pathological areas can sometimes be found pointing to the lesions beneath, but the pia should not be

stripped from those portions which are to be examined microscopically. To remove the pia an incision is made on the median surface of each hemisphere just above the corpus callosum from one extremity to the other, and the pia stripped back first from the median and then from the convex surface. The stripping is done by means of the fingers, with occasional aid from the forceps.

Section of the Brain.—There are several methods of cutting up the brain, no one of which is particularly suitable to all occasions. That method must be chosen which is most fitted to the individual case and to the use to which the tissue is to be put.

The ideal method from a neuro-pathological standpoint would undoubtedly be to harden the brain entire, and then to make serial frontal sections thin enough for microscopical purposes through the whole organ. The nearest approach to this ideal method is to harden the brain entire in formaldehyde, a process occupying ten days to two weeks (see page 347), to make thin serial sections, to mordant the sections, divided if necessary into smaller pieces, in a chrome salt (preferably by Weigert's quick method), and then to carry through a number of series from the important parts for microscopical examination. By this means the relations of the various cerebral structures and of the pathological lesions can be perfectly preserved and studied. This method can be particularly recommended for tracing degenerations in the motor tract.

If there is a noticeable focal lesion, such as a tumor or hemorrhage, it should be so incised, generally frontally or horizontally, as best to show its relations to the important cerebral tracts and ganglia. In these cases also the best results are obtained by hardening the brain entire in formaldehyde, and later making serial sections for macroscopic study or for carrying through for histological purposes. In many cases, however, it is necessary or advisable to examine the lesions in the fresh state. For instance, if it be desired to study the neuroglia-fibers, it is positively necessary to cut out thin slices of fresh tissue and to fix them immediately in

the proper solution. Often, too, the lesion cannot be or is not found except on fresh examination, or the clinician whose case it is desires to see at once the cause of certain symptoms. Under such circumstances the more ideal method must be sacrificed, and as much made out of the case as is possible in the condition in which it is left after the examination.

For the routine examination of the brain, to demonstrate

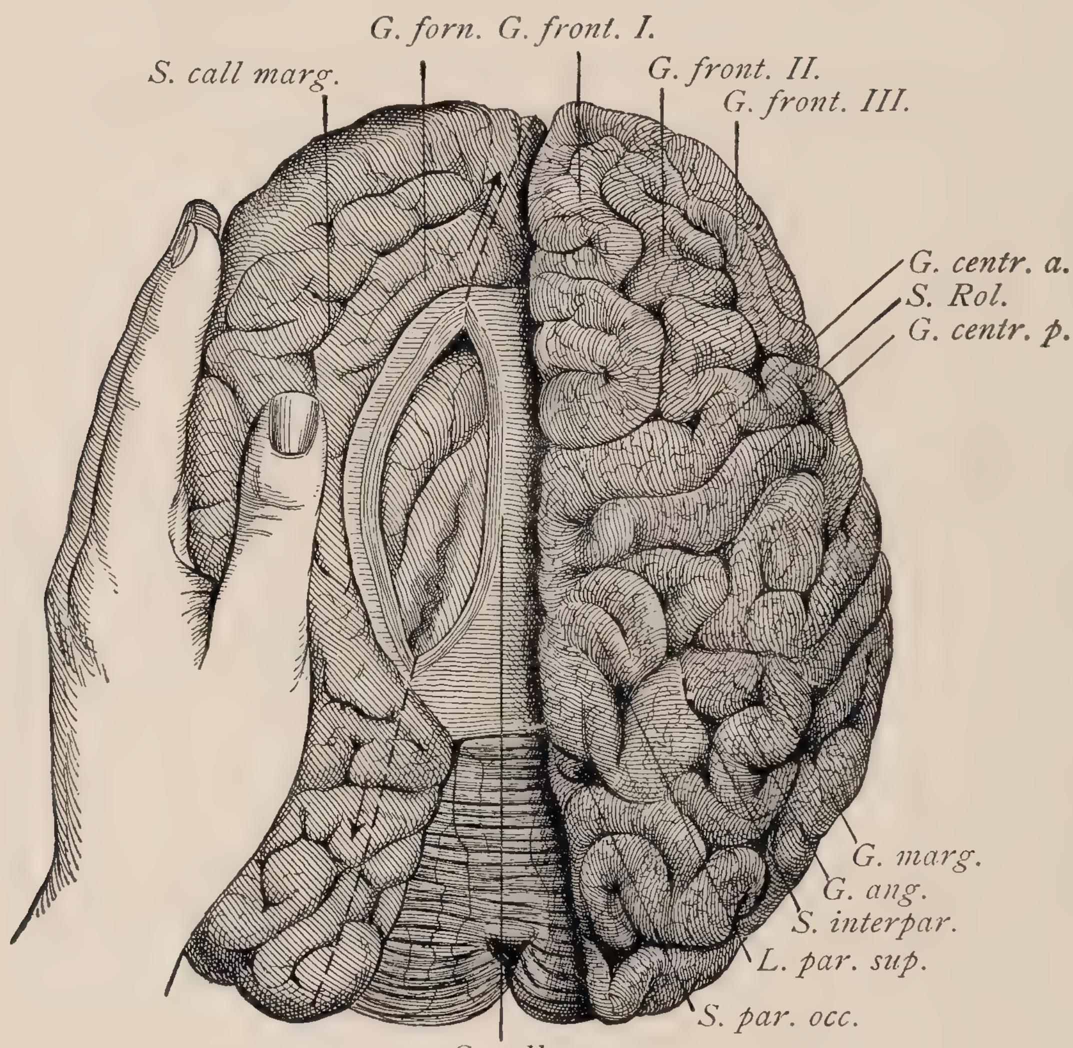

FIG. 9.—First cut in the brain (Nauwerck).

its topography and to bring to light suspected or unsuspected lesions, probably no method is more generally used than Virchow's. The objection most often made against it is that the cerebral cortex is too much cut up. In case, however, it is desired to preserve the cortex or parts of it for microscopic purposes, the longitudinal incisions after the first may be omitted, and the cortical portion, after being separated from the stem, may be cut in any way that seems advisable. In like manner, the brain-stem or any other part

may be left uncut, and hardened entire in formaldehyde for histological purposes.

Virchow's Method.—The brain is to be placed on its base in the same position as one's own. Press the hemispheres apart a little so as to expose the corpus callosum. Hold the left half of the cerebrum in the left hand with the fingers on the lateral aspect and the thumb in the longitudinal fissure. Then make an almost vertical incision with a long, slender knife through the roof of the left ventricle in its middle third, 2 to 3 mm. from the median raphé of the corpus callosum. The roof of the ventricle is to be slightly raised vertically by the thumb, so that the incision, which must not be too deep, may not injure the basal ganglia. The incision is to be continued into the anterior and posterior cornua. Then make a long incision from one end of the above cut to the other, passing just outside of the basal ganglia at an angle of about 45°. Repeat the process on the right side, turning the brain half around. Next seize what remains of the corpus callosum and fornix in the middle, lift them, and cut through from below up, passing the knife through the foramen of Munroe. The parts are then turned back, exposing the velum interpositum and the choroid plexuses. By drawing back the velum interpositum the third ventricle is uncovered.

The corpora quadrigemina are exposed by cutting transversely the right posterior pillar of the fornix and adjoining brain-substance and carrying them over to the left. Each ventricle as it is opened is to be carefully inspected and any abnormal condition of its ependyma noted. The cortex is further divided on one side, and then on the other, by holding it in the left hand and making vertical straight sections from the upper angle of the previous cut into the convex cortex, allowing the sections to fall apart, so as to avoid touching and soiling the surface with knife or fingers. Each portion thus cut represents a prism. The incisions should go well into the cortex, but not so far as to separate the different pieces. The basal ganglia are examined by means of a number of frontal sections. For this purpose the left hand is placed palm upward underneath the brain, so that as

each section is made over the tips of the fingers by one long stroke of the knife it falls forward, exposing a clean surface of which the two halves can be compared. An incision is next carried through the middle of the pineal gland, the corpora quadrigemina, and the vermiform process of the cerebellum, opening the aqueduct of Sylvius and the fourth ventricle.

Each half of the cerebellum is divided by a median hori-

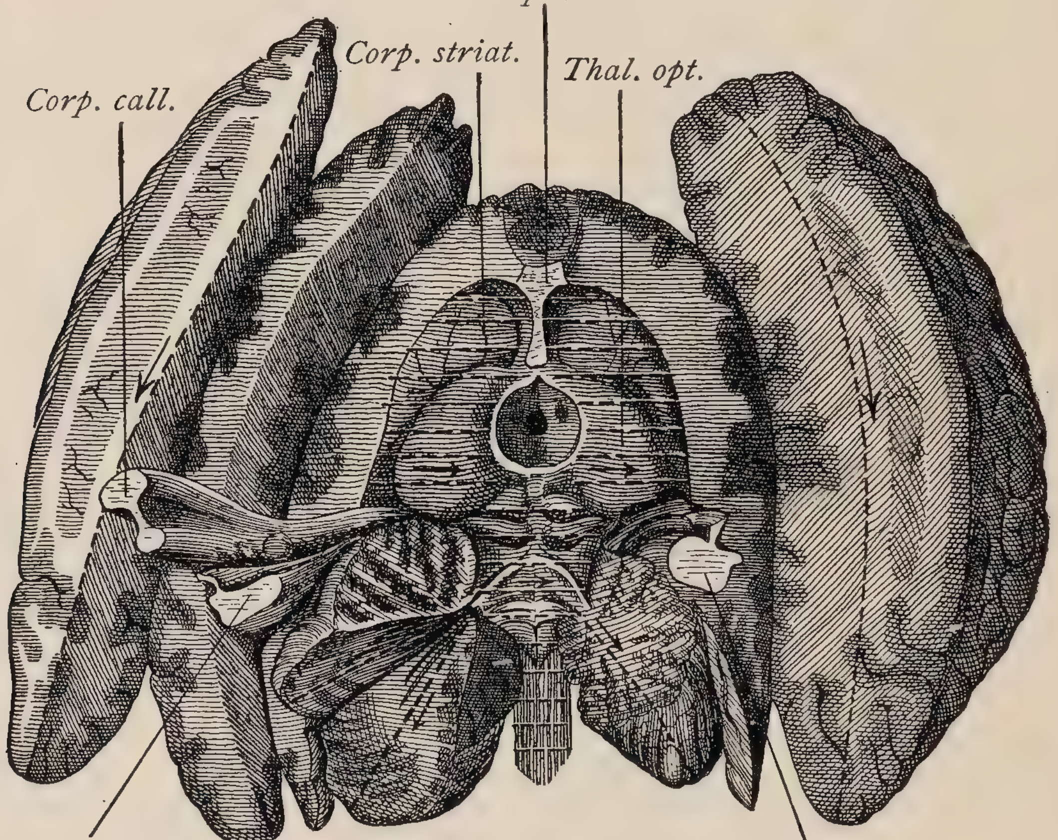

FIG. 10.—Section of the brain (Nauwerck).

zontal section into halves, and these portions are still further subdivided by a series of cuts radiating from the peduncles.

In order to make sections of the pons and medulla the brain is folded together and turned over. Several cross-sections are then made with the left hand placed beneath as in sectioning the basal ganglia.

Before making the sections it is well to remove the basilar and vertebral arteries, especially if they are calcified.

In *Pitre's method* of dissecting the brain the lateral ventricles are opened as in Virchow's method. Then the pedunculi cerebri are cut squarely across, so as to remove the pons

and cerebellum, and a longitudinal incision is carried down through the third ventricle, halving the cerebrum. Through each half of the cerebrum a series of six sections is then made parallel to the fissure of Rolando. The names of the sections and the important parts which they show are as follows:

1. The *pre-frontal section* through the frontal lobe, 5 cm. anterior to the fissure of Rolando, shows the gray and white substance of the frontal convolutions.

2. The *pediculo-frontal section* through the posterior portions of the three frontal convolutions shows the anterior extremity of the island of Reil, the lenticular and caudate nuclei, and the internal capsule.

3. The *frontal section* through the ascending frontal convolution, parallel to the fissure of Rolando, shows the optic thalamus, the lenticular and caudate nuclei, the claustrum, the external and internal capsules, the anterior portion of the descending horn of the lateral ventricle, and the island of Reil.

4. The *parietal section* through the ascending parietal convolution shows portions of the same structures as the preceding, and a transverse view of the hippocampus.

5. The *pediculo-parietal section* through the parietal lobe, 3 cm. posterior to the fissure of Rolando, shows the tail of the caudate nucleus in two places and the posterior portion of the optic thalamus.

6. The *occipital section* through the occipital lobe, 1 cm. in front of the parieto-occipital sulcus, shows simply the white and gray matter of the occipital lobe. The cerebellum, pons, and medulla are incised in the manner already described.

Removal of the Spinal Cord.—The body is to be placed face downward, with the head over the end of the table and a block under the chest. The incision is made over the spinous processes from the occiput to the sacrum. Dissect the skin and muscles back on each side, so as to leave the vertebral laminæ as bare as possible. The laminæ may be cut through by means of several instruments, of

which the double-bladed saw (Luer's rhachiotome) is perhaps the safest, at least for beginners. The single-bladed saw with rounded end is also very useful and can be thoroughly recommended. The operation can be done most quickly by biting off the spinous processes with the heavy bone-forceps and cutting through the laminæ with chisel and hammer, but there is greater danger of injuring the cord.

The numerous artifacts in the cord, reported as neuromata and heteroplasia even within very recent times by competent pathologists, would seem to indicate that the need of careful and delicate technique in the removal of the spinal cord is not yet fully appreciated.

The laminæ should be sawn nearly or entirely through in a line with the roots of the transverse processes from the third or fourth lumbar vertebra to the cervical region. The arches of the cervical vertebræ are best divided with a heavy bone-cutter, because they cannot be easily sawn, and there is sufficient room here for the points of the bone-cutter without danger of their pressing on the cord.

It is important to strike the outside limits of the spinal canal, so as to get as much room as possible for the removal of the cord. Test if the sawing be deep enough by the mobility of the spinous processes. If necessary, they can be freed by means of the hatchet-chisel and a hammer in the same way that the calvaria is loosened.

As the cord reaches only to the second lumbar vertebra, cut through between the third and fourth, free with the heavy bone-cutter the lower end of the row of the spinous processes, which are held together by their ligaments, and strip them up to the neck; then cut through the cervical arches with the bone-cutter, taking care that the point within the canal does not come in contact with the cord.

The nerve-roots are to be divided with a sharp scalpel by means of a long cut on each side of the cord. Then cut across the dura and the nerve-roots at the lower end of the exposed canal, and, while holding the dura with forceps, carefully free the cord from below up with scissors or scalpel, taking care all the time not to pull or bend the cord, be-

cause in either way artifacts may be produced. Cut the cord squarely across as high in the cervical canal as possible, so that the remaining portion may be easily removed with the brain.

Lay the the cord after removal on a flat surface and incise the dura longitudinally, first posteriorly and then in front. A series of cross-sections, usually 1 to 2 cm. apart, is made through the cord while supported on the fingers during the

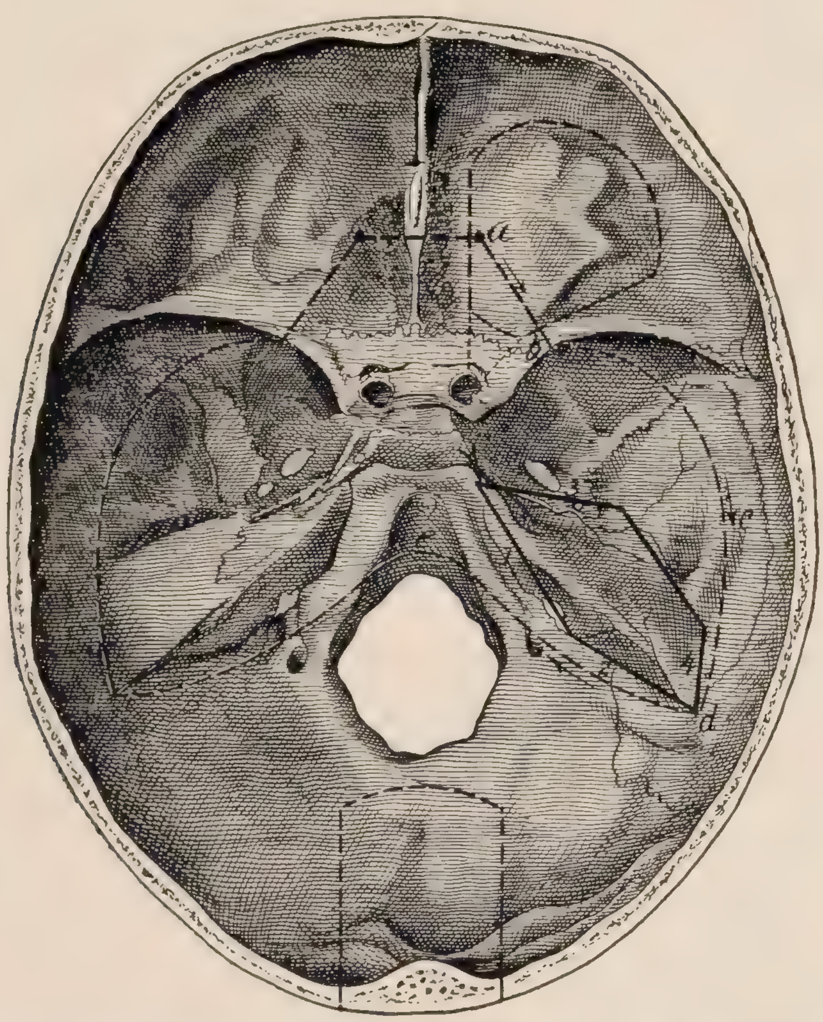

FIG. 11.—Base of skull, showing lines of incision for removing internal eye, etc. (Nauwerck).

cutting, so that the cut surfaces shall fall apart. The different segments should ordinarily be left attached to the dura, so that their position in the cord can easily be determined.

A diagnosis from the fresh, macroscopic appearances of the cord is often very difficult to make, according to the best authorities.

The Eye.—The contents of the orbit, including the posterior part of the eye, can be readily examined by chiselling

off the roof of the orbit. The posterior half of the eye can be removed by cutting around the eyeball with sharp scissors without changing the hold of the forceps on the sclera. If done quickly, the retina remains quite well spread out. The anterior half of the eyeball is to be propped in place by a plug of cotton dipped in ink or in a solution of permanganate of potassium.

The Ear.—The middle ear can be exposed by chipping off with a chisel its roof, which lies in the middle of the petrous portion of the temporal bone. The roof can also be very easily bitten off with the heavy bone-cutters. If, however, it be desired to examine the ear more carefully by means of a section through the external meatus and the middle ear, it will be necessary to remove the whole of the petrous bone. For this purpose the incision behind the ear must be carried back along the anterior edge of the trapezius muscle halfway down the neck. Then the skin-flaps, including the external ear and the underlying tissues, must be dissected back for some distance on each side of the incision. Two converging incisions are then to be sawn, the anterior passing through the root of the zygomatic arch, the posterior just back of the sigmoid sinus, so as to come together at the apex of the pyramid of the petrous bone, or, better still, to meet in the foramen magnum. An ordinary chisel and a hammer or mallet will be found very convenient for freeing the petrous bone after the incisions have been sawn.

In the examination of the petrous bone after it has been removed the first step is to chisel off the tegmen tympani so as to get a view of the middle ear. Next remove the lower wall of the external meatus, so as to expose the outer surface of the membrana tympani. Finally divide the petrous bone with a fine hair-saw by an incision starting in at the styloid process and coming out at the carotid canal, parallel to the crest of the pyramid of the petrous bone.

This incision divides the cavum tympani into halves. In the lateral half can be seen the membrana tympani with the hammer and the anterior half of the mastoid cells. In the

median half are the labyrinthine wall of the cavum tympani with the stapes and the posterior half of the mastoid cells. It is best to remove the anvil before sawing through the bone. The Eustachian tube can be easily exposed by starting from its termination in the middle ear.

The Naso-pharynx.—Although a fair view of the nares and pharynx can be obtained by chiselling off the portion of the base of the skull lying over them, the method does not begin to offer the satisfactory view that can be obtained by the method of Harke,[1] a method which is not so difficult as might at first sight seem, and which consists in halving the base of the skull by a longitudinal incision. To do this the original incision in the scalp must be extended on each side over the mastoid processes and along the anterior edge of the trapezius muscle to a point below the middle of the neck. Then the posterior flap and the underlying muscles must be freed from the occipital bone and the upper portion of the cervical vertebræ. In like manner, the anterior flap must be dissected from over the root of the nose and the upper edge of the orbits, and be drawn down over the face. Then flex the head strongly forward and saw through the occipital bone and the base of the skull, dividing the occipital and frontal bones, the sella turcica, the cribriform plate, and the basilar process into equal halves. Anteriorly, it is well to go a little to the left or right, so as not to injure the nasal septum.

The next step is to cut the pachymeninx and the apparatus ligamentosis between the anterior edge of the foramen occipitale magnum and the processus odontoideus, as well as the inner side of the atlanto-occipital joint from within. Then the two halves of the skull are to be drawn forcibly apart. The nasal bones, the hard palate, and the alveolar process of the upper jaw break, and the two halves of the base of the skull open like a book, revolving around an axis which passes through the joint of the lower jaw and the atlanto-occipital joint.

If the foramen occipitale magnum offer too much resist-

[1] *Berliner klin. Wochenschrift*, 1892, No. 30.

ance, break through it with a chisel, and also if necessary through the anterior and posterior arches of the atlas.

It is now easily possible to inspect the sinus sphenoidales, the nasal septum, the frontal sinuses, and the nasal passages. The antrum of Highmore is easily opened with forceps and a pair of bone-shears.

After the operation the two halves of the base of the skull are brought together, and wired if necessary. When the skin-flaps have been replaced all evidence of the operation is covered up.

Examination of New-born and Very Young Children.—1. The head is preferably opened by the method given on page 53.

2. According to Nauwerck, the spinal canal can be opened by dividing the vertebral arches with strong scissors.

3. The umbilical cord, if present, and the umbilical arteries demand close attention in children who have lived a few days or weeks, for the purpose of determining if infection has taken place at that point. Nauwerck advises a modification of the primary long incision. A little above the umbilicus it should divide into two diverging incisions running to the pubes. In this way a triangular flap is left containing the umbilical arteries, while from the upper end is given off the umbilical vein. The vessels may be ligated or opened at any point that seems advisable.

4. Anomalies of circulation should be looked for in all "blue babies." The closure or non-closure of the *ductus Botalli* (arteriosus) is best determined *in situ* by dissecting off the thymus and opening up the pulmonary vein in the middle of its anterior surface. The cut may be extended downward, if desired, through the pulmonary valve and the wall of the right ventricle. The duct lies in the median line of the pulmonary artery, a little above its division into its two main branches. A small probe can be passed through it into the aorta. The condition of the *foramen ovale* between the auricles is easily examined.

For other anomalies of the circulation it will usually be found most satisfactory to remove the thoracic organs in

mass, so as to be able to open up the heart and the vessels given off from it before any of the vessels have been severed from their connections.

5. In medico-legal cases especially it is important to determine whether or not a child has breathed. The main steps of the process are as follows:

(*a*) Position of the diaphragm before the chest is opened. When the lungs are fully distended it is at the fifth or sixth rib on the right and at the sixth rib on the left. When the lungs contain no air or are but partially distended the diaphragm reaches to the fourth rib.

(*b*) Ligate the trachea above the sternum before opening the thorax.

(*c*) After examining the heart, etc., divide the trachea above the ligature and remove the thoracic organs in one piece.

(*d*) Dissect off the thymus gland and the heart, and place the lungs in a large dish of clear cold water to see if they will float or not.

(*e*) Incise the lungs and notice if they crepitate; squeeze the lung-tissue gently, and see if bubbles of air mingle with the blood on the surface, or squeeze the lung beneath water and observe if bubbles of air rise to the surface. Decomposition may give rise to gas in the lungs.

(*f*) Divide the lungs into lobes, and then into small pieces, and determine if any of them will float.

Table of the Weight and Length of the Fetus at each Month of Gestation (from v. Hecker, cited by Nauwerck).

Time in months.	Weight.	Length.
2	4 gr.	2.5–3 cm.
3	5–20 "	7–9 "
4	120 "	10–17 "
5	284 "	18–27 "
6	434 "	28–34 "
7	1218 "	35–38 "
8	1549 "	39–41 "
9	1971 "	42–44 "
10	2334 "	45–47 "

6. The long bones should be incised, so as to expose the

epiphyseal line, which should be examined for evidences of congenital syphilis. The ends of the femur and tibia at the knee are usually chosen. For making the incision a fine hair-saw is preferable to a knife, because the latter often causes the bone to break apart at the epiphyseal line.

The age of the fetus in months can be determined after the fifth month by dividing the length in cm. by 5.

Weight of Organs in a New-born Child.

Brain	380 gr.	(Bischoff).
Thymus	14 "	(Friedleben).
Heart	20.6 "	(Thoma).
Lungs	58 "	
Spleen	11.1 "	
Kidneys together	23.6 "	(Thoma).
Testicles	.8 "	
Liver	118 "	

Restitution of the Body.—After an autopsy is finished it is necessary to put the body into such a condition that no evidence of the operation will be noticed except on careful inspection. All fluids should be removed from the cavities. Organs not required for further examination should be replaced. The brain is placed in the body-cavity because it is usually impossible to restore it to the skull. The best material for filling up the cavities is fine sawdust. It packs easily and smoothly, absorbs well, keeps the needle dry so that it does not slip, and does not interfere with sewing like oakum, which gets into the stitches. In private autopsies any make-shift, such as bran, newspapers, or cloth, must be employed. If the pelvic organs have been removed, stuff the pelvis tightly to prevent leakage. The cranium may be left empty, although it is usually better to pack a little sawdust or other material into the base of the skull and the upper part of the spinal canal to prevent leaking. Sometimes it is advisable to fill the cranial cavity with sand or sawdust wrapped tightly in a cloth, of which the edges are brought together and twisted so as to crowd the material into a compact mass. If the thoracic cavity is well packed with sawdust, the sternum will stay perfectly in place without being sewed.

If part of the vertebral column has been removed, a stick or heavy iron rod should be run into the spinal canal above and below, so as to stiffen the body and hold it in position while it is filled about half full of plaster of Paris. After this has set there is little danger of the body losing its form.

In sewing up the body-cavity, begin at the neck. Use a piece of twine a little over one and a half times the length of the incision. Take one stitch and fasten the end with a simple knot or with a surgeon's knot. Turn the loose end in under the skin. Hold the attached end of the twine taut with the left hand about 8 to 10 cm. from the line of incision. The needle is then passed from within outward through the edge of the flap and in a diagonal line from below upward. The stitches should be from 1 to 2 cm. apart, and about the same distance from the edge of the flap. The object of keeping the end of the twine taut is to keep the sutures tight and the edges of the flaps up so that the needle can be thrust in easily.

Arrived at the lower end of the incision, take two buttonhole stitches and draw them tight. Then take a long stitch off to one side and cut the twine close to the skin, so as to bury the end of it deeply and securely.

If in removing the calvarium the precaution is taken to crack at least a part of the inner table with the chisel and hammer, projecting pieces of bone are usually left which interlock and hold the calvarium snugly in position when it is replaced. It is further fastened by sutures on each side through the fascia of the temporal muscle. It is always more difficult to sew up the incision in the scalp than the one in the body, especially when the hair is long. Care should be taken to bury the ends of the suture securely.

The skull of a child is so thin that it is usually best to wire the calvarium in place or fasten it by means of double tacks, otherwise it may slip out of place after the scalp has been sewed up.

Slee's ingenious method deserves mention. The usual saw-cuts in the skull over the ear are allowed to cross each other, so that slits about an inch long are formed in the tem-

poral bone. An ordinary roller bandage is stretched across the skull and crowded edgewise into the slits. Then the calvarium is replaced and the ends of the bandage are tightly overlapped over the vertex and secured by pins.

List of Publications on Post-mortem Technique.

1. Virchow, Sektionstechnik, 4 Aufl., 1893.

2. Orth, Pathologish-anatomische Diagnostik, VI Aufl., 1900.

3. C. Nauwerck, Sektionstechnick für Studierende und Aerzte, V Aufl., Jena, G. Fischer, 1912.

4. Chiari, Pathologisch-anatomische Sektionstechnik, Berlin, II Aufl., 1907.

5. G. Hauser, Die Zenkersche Sektionstechnik, Jena, G. Fischer, 1913.

PART II.

BACTERIOLOGICAL METHODS.

I. CULTURE-MEDIA.

CULTURE-MEDIA consist of various nutritive substances, either liquid or solid, in or upon which bacteria will grow and multiply, and are, as a rule, contained in test-tubes ready for use.

The nutritive material in these test-tubes must be free from living bacteria—*i. e.* "sterile"—and must be kept so until used. This is accomplished by inserting a stopper of raw cotton into the mouth of each test-tube to exclude the entrance of bacteria from without, and then subjecting the tubes and their contents to the sterilizing action of live steam for the purpose of killing any bacteria which may have gained access to the medium during its preparation.

The Preparation of Test-tubes.[1]—*New test-tubes* should be washed in a very dilute solution of nitric acid (2–5 c.c. of the commercial nitric acid to the liter of water), then thoroughly rinsed in water and allowed to drain until dry or nearly so. The object of the use of the nitric acid is to remove any free alkali which may be present in the new tubes.

Old test-tubes containing culture-media, after removal of the cotton stoppers, should be boiled for from half an hour to one hour in a solution of common soda (4–6 per cent.). This treatment not only destroys bacteria, but it also loosens and liquefies the material in the tubes, so that it may be easily removed with the aid of a test-tube brush and plenty of water.

[1] Test-tubes of the size known as 6 × ¾ in. are recommended.

When all the material has been removed from the test-tubes in this way, they are to be rinsed in clean water, then in the dilute nitric acid of the strength above indicated for the new test-tubes, and finally again rinsed in clean water, after which they are to be allowed to drain until dry or nearly so.

The test-tubes thus prepared are next to be provided with stoppers of raw cotton (not absorbent cotton), which are to be inserted into the mouths of the tubes for a distance of about 3 cm., and should fit the walls of the tubes smoothly. The stoppers should not be packed in nor fit too tightly, but be just firm enough in position to easily sustain the weight of the tube when it is lifted by the projecting portion of the cotton.

The stoppered tubes are then to be packed into a square wire basket which fits into the hot-air sterilizer,[1] and heated in this, with the door closed, until the temperature reaches about 150° C. The object of this heating is not to sterilize the tubes and cotton stoppers, but to mould the stoppers to the shape of the test-tubes, so that they can readily be replaced when removed in the subsequent filling of the tube with nutritive material. In packing the tubes into the square wire basket as many as possible should be placed with the cotton stopper uppermost, and the remainder of the space in the basket above the tubes may be filled with tubes placed on their sides.

PREPARATION OF CULTURE-MEDIA.

Bouillon.—Formula for 1000 c.c.:

Lean beef,	500 grams;
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.c.

500 grams, or about 1¼ pounds, of lean beef, finely minced, are thoroughly mixed with 1000 c.c. of ordinary tap-water

[1] See any dealer's catalogue of bacteriological apparatus.

and the mixture is then boiled in a saucepan over the gas stove[1] for about half an hour. It is next filtered through filter-paper to obtain the clear infusion of the beef, free from the coagulated albumin and shreds of tissue. This clear beef-infusion is then turned back into the saucepan, which should be clean, and to it are added 10 grams of pepton (Witte), 5 grams of sodium chlorid, and sufficient water to make the total volume of the mixture 1000 c.c. The volume of 1000 c.c. may be indicated with sufficient accuracy by a mark previously made on the inner surface of the side of the saucepan. The mixture is next to be boiled until all these substances are dissolved, stirring frequently with a glass rod, and is then to be neutralized, for it has a decidedly acid reaction from the acid of the meat.

The neutralization is important and requires care (see also p. 84). The reaction required is that of a very faint alkalinity, as is shown by the production of a blue color on red litmus paper, while no change is produced on the blue litmus paper. In neutralizing, a 10 per cent. solution of caustic soda is added, a few c.c. at a time at first, and later, two or three drops at a time, while the mixture is kept boiling, the reaction being tested between each addition of alkali after thorough stirring with a glass rod.

The test of the reaction is best made by placing a drop of the mixture on a piece of litmus-paper by means of the glass rod and then moistening the paper at the water-faucet. In this way the best judgment can be formed of changes in the color of the paper. If the mixture becomes too alkaline, dilute hydrochloric acid is to be added to correct this.

When the proper reaction has been obtained the mixture is to be filtered through filter-paper into a flask, and sufficient water added to bring the volume of the filtrate up to 1000 c.c., thus replacing the loss by evaporation. The filtrate in the flask is now *bouillon*. If the bouillon be heated to the boiling-point, it will usually become more or less

[1] In the preparation of culture-media some form of gas stove is preferable to a Bunsen burner.

clouded by a precipitate of phosphates. As a rule, subsequent heatings do not cause any further precipitations. Therefore it is advisable, if it is desired to obtain perfectly clear bouillon, to steam the flask containing the freshly prepared bouillon in the steam sterilizer for about half an hour, and then, if the bouillon be clouded, to again filter, so that the subsequent sterilizations in the test-tubes will not cause precipitates.

The finely minced beef may be obtained in the shops under the name of Hamburg steak, or it may be very readily prepared with the aid of a meat-grinder.

The usual directions for the preparation of bouillon require that the mixture of the minced meat and water be allowed to stand over night in a cool place before boiling. In our experience this is not necessary.

For bouillon cultures the bouillon is run into test-tubes, each tube being filled to a depth of about 4 cm., and sterilized immediately and on the two following days, according to the general directions given on page 88, after which it is ready for use.

Bouillon may also be made as above indicated by using three grams of Liebig's extract of beef to the liter, instead of the beef-infusion.

Glucose or Dextrose Bouillon.—Formula:

Glucose or dextrose (dry),	10	grams;
Lean beef,	500	"
Or extract of beef,	3	"
Pepton,	10	"
Sodium chlorid,	5	"
Water,	1000	c.c.

This medium is identical with the preceding, except that it contains 10 grams of glucose to the liter (1 per cent.) in addition to the other ingredients. The preparation of glucose bouillon is the same as that of plain bouillon, the glucose being added with the pepton and sodium chlorid.

Agar-agar (plain).—Formula for 1000 c.c.:

Agar-agar,	15 grams;
Lean beef,	500 "
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.c.

Agar-agar is essentially bouillon in which agar-agar has been dissolved so that a transparent jelly is formed. The function of the agar-agar is merely to give the medium the property of becoming liquid when heated and solid when cool; it is not nutritive. The nutritive substances are in the bouillon.

To make one liter, 15 grams of agar-agar are placed in the clear beef-infusion, made as described on p. 72 and boiled for one hour in a saucepan.[1] The agar-agar dissolves slowly, and continuous boiling is necessary to ensure its subsequent filtration. Before boiling, about 200 c.c. of water should be added to compensate for evaporation, and later, as the level of the liquid falls, more water should be added from time to time. It is well to have some mark on the side of the saucepan which will indicate the level of a liter. When the boiling is nearly finished, 10 grams of pepton, 5 grams of sodium chlorid, and sufficient water to make a volume of one liter are added to the mixture. The mixture is then neutralized, as described for bouillon, while still boiling.

After the boiling is completed the saucepan is to be placed in cold water until the temperature of its contents falls to about 60° C., as shown by the thermometer, the cooling

[1] If an autoclave (see p. 89) be available, it may be used very conveniently in hastening the solution of agar-agar in the meat-infusion. For this purpose the mixture of finely fragmented agar-agar and the beef-infusion should be placed in a Florence flask. When the temperature of the interior of the autoclave has reached about 120° C. or when the gauge shows a pressure of two atmospheres, the heat should be turned off and the apparatus allowed to cool to about 100° C. before opening. The mixture is then transferred to a saucepan and the preparation proceeded with as above indicated.

being facilitated by stirring with a glass rod. When this temperature is reached, an egg is beaten into the mass and the saucepan with its contents replaced on the stove, where it is slowly brought to boiling and boiled for about ten minutes. The object of the adding of the egg is to clarify the medium. It is then filtered, boiling hot, through wet folded filter-paper into a flask. A funnel with corrugations on its sides is best to use. With this the folding of the filter-paper is not necessary.

In order to save time, it is best to use two filters and two flasks at once, for the filtration rapidly becomes slow as the mass cools, and several heatings of the residue on the filter are necessary. As soon as the filtrate begins to appear slowly, drop by drop, the mass remaining on the filter should be turned back into the saucepan—which can best be done by making a hole in the bottom of the filter with the glass rod—and brought again to boiling. While boiling hot it is again poured on a fresh filter. This preparation of fresh filters and reheating may have to be repeated several times before all of the mixture is filtered. The filtration may also be carried on in the steam sterilizer to prevent the cooling of the medium.

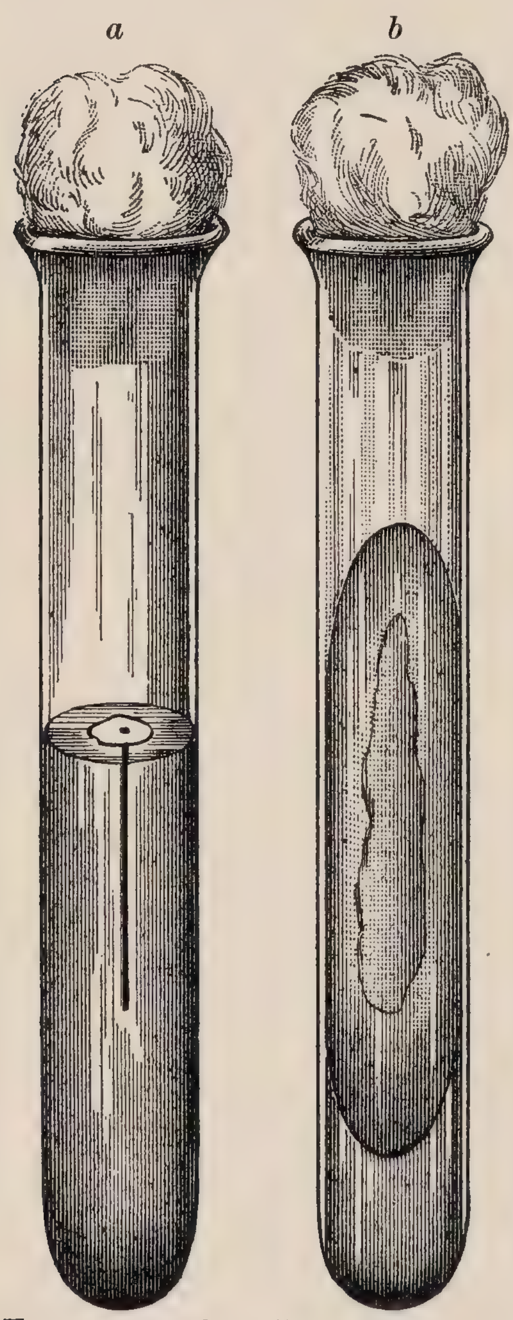

FIG. 12.—"Stab" culture (*a*); "slant" culture (*b*).

When the amount of coagulated egg-albumin and medium remaining on the filter does not exceed a volume of 50–100 c.c., the filtration may be considered complete. To the filtrate, which is now agar-agar, is next added sufficient water to make up the loss by evaporation, and the medium is then to be run into test-tubes and sterilized, as described on page 88.

In view of the difficulty of filtering agar-agar, it has been proposed to avoid this operation by placing the fluid medium in a sedimenting vessel, such as a large funnel, with closed apex. The solid particles settle to the bottom if the medium be maintained in a fluid condition in the steam sterilizer for a certain length of time. When the medium has become solid it is turned out of the vessel as a cast, and the bottom portions, containing the sediment, cut off from it and rejected. The remaining portion will be found clear enough for most purposes and may be melted up at once for distribution in tubes, or if it now be desirable to further clarify it, it may be melted up and filtered as described above. It will be found to filter more readily than before.

Precipitates of phosphates in the medium frequently occur after the first sterilization, but if these be removed they do not usually appear again in subsequent heatings. Therefore, if it be desirable to obtain a very clear agar-agar, it is well to place the flask containing the freshly-prepared medium in the steam sterilizer for half an hour, and then filter again to remove any precipitate which may have appeared. The subsequent sterilization in the test-tubes will then cause no precipitation.

In filling the test-tubes it is customary to fill some tubes to a depth of about 3 cm. and others to a depth of about 5 cm. After the complete sterilization of the medium in the tubes as described on page 88, the first-mentioned tubes are placed on their sides with their mouths slightly elevated while the medium is still fluid, so as to form, after solidification, a slanting surface extending from near the bottom of one side of the tube to about half the length of the tube on the opposite side. The solidification of the agar-agar takes place in a short time, and as soon as it occurs the tubes are ready for use, this form of culture being known as a "slant" tube or culture (Fig. 12, *b*). It is well, however, to allow the tubes to remain in their slanting position for a day or two to permit the medium to become more or less adherent to the walls of the tube, and thus avoid its tendency to slide downward when the tubes are placed in the upright position.

The tubes filled to a depth of 5 cm. are to be allowed to cool and solidify while in an upright position, and the form of culture-tube thus obtained is called a "stab" culture (Fig. 12, *a*), because the medium in the tube is inoculated for culture purposes by inserting an infected platinum wire into its depths.

Glucose Agar-agar.—Formula for 1000 c.c.:

Glucose (dry),	10 grams;
Agar-agar,	15 "
Lean beef,	500 "
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.c.

This medium differs from plain agar-agar only in the addition of 10 grams (1 per cent.) of glucose. The glucose should be obtained in the form of solid masses, not as a thick fluid, and it is to be added with the pepton and sodium chlorid. In short, glucose agar-agar is made with glucose bouillon in identically the same manner that plain agar-agar is made with plain bouillon.

Lactose-litmus agar-agar consists of plain agar-agar to which has been added 2 or 3 per cent. of lactose and sufficient litmus tincture to give it a pale-blue color.

Glycerin Agar-agar.—Formula for 1000 c.c.:

Glycerin, c. p.,	60 c.c.;
Agar-agar,	15 "
Lean beef,	500 grams;
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.c.

This medium is prepared by adding to plain agar-agar after its final filtration, and before running it into the test-tubes, 60 c.c. (6 per cent.) of glycerin c. p., and mixing thoroughly.

Gelatin (plain).—Formula for 1000 c.c.:

Gelatin,	100 grams,
Lean beef,	500 "
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.c.

Gelatin is essentially bouillon in which gelatin has been dissolved, so that a transparent jelly is produced which is solid at ordinary temperatures and fluid when slightly warmed. To prepare one liter, 100 grams (10 per cent.) of *golden seal French gelatin* are dissolved in a liter of the hot bouillon which has been heated to boiling in a saucepan. When the gelatin is thoroughly dissolved the mixture is boiled for about five minutes, and the marked acidity of the gelatin then carefully neutralized by the addition of caustic soda, in 10 per cent. solution, to a very faint alkalinity, as has been described in the preparation of bouillon. As in the case of agar-agar, the mass is then cooled to 60° C., an egg beaten into it, then gently heated again to boiling, and boiled about ten minutes, when it is to be filtered through a wet folded filter into a flask. Gelatin usually filters fairly rapidly, but time may be saved by using two filters at once. When filtered it is to be run into test-tubes and sterilized, as described on page 88. It is used both in the form of "slant" and "stab" cultures, as in the case of agar-agar (see page 77).

In the preparation of this medium it is important to subject it as little as possible to the boiling temperature, for prolonged exposure to this destroys its power of solidifying. Therefore in sterilizing, gelatin tubes should never be allowed to remain exposed to live steam longer than twenty minutes. It is also important to apply the heat slowly during the process of heating after the addition of the egg above mentioned, in order to avoid "burning."

Glucose or Dextrose Gelatin.—Formula for 1000 c.c.:

Glucose or dextrose,	10 grams;
Gelatin,	100 "
Lean beef,	500 "
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.cm.

This medium is essentially gelatin dissolved in glucose bouillon (see page 74), and is prepared in the same manner as the plain gelatin, except that glucose bouillon is used instead of plain bouillon.

Blood-serum (Löffler's Mixture).—Formula:

Glucose bouillon (see page 74),	1 part;
Beef blood-serum,	3 parts.

This culture-medium consists of a mixture of the blood-serum of the bullock and glucose bouillon, which is run into test-tubes and coagulated by heat in such a way as to form a slanting surface for culture purposes—*i. e.* it is used in the form of "slants."

The *blood-serum* is collected at the slaughter-house in tall glass jars of the capacity of a gallon or more. These jars should be thoroughly clean, but sterilization is not necessary.

The blood which is obtained by the Jewish method of slaughter—viz. by severing the carotid artery—is the best for the purpose, because it clots more readily. As the blood runs from the vessels of the animal it is received in the glass jar, and immediately placed in a cool place for twenty-four to forty-eight hours to allow it to clot and the serum to separate. All unnecessary agitation of the fresh blood should be avoided, as this interferes with its proper clotting. It is well to inspect the blood after a few hours, and gently loosen with a clean glass rod any adhesions which the clot may have formed to the wall of the jar, thus allowing the clot to more readily contract and squeeze out the serum

from its meshes. After about twenty-four hours the serum is removed by the aid of a clean pipette and brought to the laboratory. If the clot is in good condition, more serum will appear after another twenty-four hours, and if necessary this also may be used.

The presence of red blood-corpuscles in the serum is of little importance. *Three parts* of the beef blood-serum thus obtained are to be thoroughly mixed with *one part* of glucose bouillon (*vide supra*), convenient quantities being 900 c.c. of *blood-serum* and 300 c.c. of *glucose bouillon.*

This mixture is then run into test-tubes as described on page 86. The quantity run into each test-tube should be sufficient to fill it to a depth of about 3–4 cm. The tubes containing the requisite amount of the mixture are next subjected to the action of heat while in a slanting position, so that the mixture in the tubes may become solid or coagulated, and so offer a smooth slanting surface for culture purposes extending from a point near the bottom of the tube to about halfway up the opposite side or higher.

The coagulation is effected either in the hot-air sterilizer by packing the tubes on their sides, the proper slant being secured by means of strips of cardboard placed between the layers of tubes, or better, in the blood-serum coagulator which may be obtained from dealers in bacteriological apparatus.

If the hot-air sterilizer is employed, the temperature should not exceed 90° C. nor fall below 85° C., and the door should be kept closed. It is optional whether the sterilizer be packed full of tubes or only a few layers of tubes be coagulated at a time, with careful watching to avoid overheating. In the former case two or three hours will be required to firmly coagulate the tubes in the middle layers, while the lower layers may be overheated. To avoid this overheating of the lower layers, a false bottom or one or two layers of empty tubes may be employed.

The blood-serum coagulator is much more convenient and gives much more satisfactory results. The temperature

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of the interior should be kept at about 95° C. To save time in heating, the apparatus may be filled with hot water from the hot-water faucet.

Whichever apparatus is employed for coagulation, it is of the utmost importance that the coagulation of the mixture be a thorough one, and that the medium in the tubes becomes firm and solid, otherwise bubbles and cavities will form in it and destroy its smooth surface when it is subjected to the subsequent steam sterilization. When the tubes are firmly coagulated they are to be packed with the cotton stopper uppermost in a round wire basket and sterilized by steam three times, as indicated on page 88, after which they are ready for use.

This method of preparing blood-serum tubes is very different from the one usually described, a most tedious and time-consuming procedure, requiring a high degree of technical skill, by which it is practically impossible to make use of blood-serum tubes for ordinary purposes.

With the method here detailed we think that the best culture-medium for the routine examination of pathological material is obtained. It is preferred by us for various reasons, chief of which are as follows:

First, the ease and facility with which it can be prepared, especially when a proper coagulating apparatus is available.

Secondly, the greater and more rapid growth of certain important pathogenic bacteria upon it than upon ordinary media.

In the method usually described the serum (which should be clear or free from blood-corpuscles) is obtained under all aseptic precautions, is carefully mixed with sterile glucose bouillon in the proportions given above, and the mixture then run into sterile test-tubes. During all the manipulations precautions are necessary to avoid contamination, the serum never being allowed to come in contact with any object which is not sterile, and exposure to the air during the processes of transference from one vessel to another avoided as much as possible.

The mixture now being in test-tubes, it is subjected for one hour on each of five successive days to a temperature of 68° to 70° C. in a chamber provided with a water-jacket. This tem-

perature is sufficient to kill the vegetative forms of any bacteria which may be in it, but does not coagulate the medium. The intervals between the sterilizations are for the purpose of allowing any spores to develop into the vegetative form and thus become susceptible to the destructive action of heat.

After the fifth sterilization the medium is solidified in the tubes in the form of "slants" by slowly raising the temperature of the chamber to about 80° C., and keeping the tubes at this temperature for several hours. In solidifying the great object is to obtain a gelatin-like, fairly transparent medium and to prevent opacity. To attain this it is necessary to proceed very carefully with the heating and avoid overheating or too rapid heating, the tubes being inspected from time to time and removed from the chamber as soon as their contents have the proper consistency. When gelatinized the tubes are placed in the incubator for twenty-four hours to determine whether they are sterile, after which they are ready for use.

The blood-serum medium produced by this older method is especially suited for the cultivation of certain pathogenic bacteria —for instance, the bacillus tuberculosis and the bacillus diphtheriæ —but we do not think that its superiority in this respect over the more readily prepared, firmly coagulated form above described is sufficiently marked to compensate for the great difficulties in its preparation.

Litmus-milk is a form of culture-medium used for determining certain of the physiological properties of bacteria. It consists of cow's milk which has been colored blue by litmus and containing a minimum amount of cream. A pint or so of strictly fresh milk is placed in a flask and steamed in the steam sterilizer for about half an hour. When it is removed it will be found that most of the cream has collected at the surface, and it is then easy to draw off the milk from the deeper layers with a pipette into a separate flask. To the milk from which most of the cream has been thus removed is added sufficient of an aqueous solution of litmus (freshly filtered) to give it a pale-blue color. The colored milk is then run into test-tubes (5 cm. deep in each tube) and sterilized, as indicated on page 88, after which it is ready for use. It is of great importance that the milk be fresh. If it is not, it may contain spore-bearing bacilli which it is practically impossible to kill by the steam sterilization.

Potato-cultures according to Bolton.—Potatoes—pref-

erably old ones—are first washed to remove all the coarser particles of soil, and then solid cylinders are cut out of them with a cork-borer or apple-corer. These cylinders should be of a suitable diameter to fit into the test-tubes used for other culture-media, and should be about 5 cm. long. They are then cut longitudinally in an oblique direction with a sharp knife, so that a smooth slanting surface is produced, beginning near one end and extending diagonally to the other end. The pieces of potato thus prepared are next to be washed in running water over night. After washing, each piece is placed in a test-tube, the larger end resting on the bottom of the tube, a few drops of water being added to prevent drying, and then sterilized as indicated on page 88. If desired, a small piece of glass rod may be placed in the bottom of the tube to elevate the potato above the few drops of water (Fig. 13).

FIG. 13.—Potato-culture.

Dunham's Pepton Solution.—Formula for 1000 c.c.:

Pepton,	10 grams;
Sodium chlorid,	5 "
Distilled water,	1000 c.c.

The pepton and sodium chlorid are dissolved by boiling and the mixture filtered. The clear filtrate is then run into test-tubes, each test-tube being filled to a depth of 5 cm., and is to be sterilized as indicated on page 88, after which it is ready for use.

The Adjustment of the Reaction of Culture-media by Titration.—Because comparatively small variations in the reaction of culture-media may have a marked effect upon the morphology and mode of growth of bacteria grown upon them, a more exact adjustment of their reaction than is possible with litmus paper is desirable. This is especially

important for media used for the cultivation of the bacteria of water, of soil, and of the air. For ordinary purposes of cultivation of bacteria, especially of the pathogenic forms, the adjustment of the reaction with litmus paper, as elsewhere described, if carefully done, will be found to be sufficient.

The more exact method of adjusting a reaction is one of titration with phenolphthalein as an indicator. The method is as follows: When the culture-medium, whether it be bouillon, agar-agar, or gelatin, has been neutralized with the aid of litmus paper, made up to the proper volume, and when it is all ready for filtering, as described elsewhere, 5 c.c. of it are transferred by means of a pipette to a 6-inch porcelain evaporating dish; to this 45 c.c. of distilled water are added, and the 50 c.c. of fluid are boiled for three minutes over a flame to expel any carbon dioxid which may be present.

Next, 1 c.c. of a 0.5 per cent. solution of phenolphthalein in 50 per cent. alcohol is added to the mixture in the dish, and immediately after this enough of a *twentieth normal* solution of sodium hydroxid is cautiously run into the dish, from a burette, to produce a pink color in the mixture. The judgment of the proper color which indicates that sufficient alkali has been run in requires some practice. The color to be obtained is a bright pink. The appearance of the proper pink color is preceded by a pinkish darkening of the fluid which may deceive the inexperienced.

The quantity of the twentieth normal sodium hydroxid solution required to effect this result is then read off from the burette. The number of cubic centimeters required denotes the percentage by volume of a *normal* solution of sodium hydroxid which would be required to make the total volume of culture-medium neutral to phenolphthalein. That this is so will be apparent after a simple calculation.

The reaction recommended by the Bacteriological Committee of the American Public Health Association as a standard to which culture-media should be adjusted is such that 1.5 per cent. of a *normal* solution of sodium hydroxid would be required to be added to the medium to make it

neutral to phenolphthalein. This reaction corresponds closely to a faint alkalinity toward litmus, for the neutral point of phenolphthalein is not identical with that of litmus. The adjustment of the reaction to this standard is effected by adding to the bulk of the culture-medium sufficient normal sodium hydroxid solution or normal hydrochloric acid solution.

For example: If the titration shows that 5 c.c. of the medium requires 1.9 c.c. of the twentieth normal solution of sodium hydroxid to make it neutral to phenolphthalein, then the total mass of the medium will require the addition of 1.9 per cent., or 19 c.c. for a liter, of a normal solution of sodium hydroxid to make it neutral; but the reaction required is such that 1.5 per cent. of a normal solution of sodium hydroxid should be required to make it neutral. Therefore, 0.4 per cent, or 4 c.c. for a liter, of a normal solution of sodium hydroxid should be added to the main mass of the medium.

When the calculated amount of normal solution has been thoroughly mixed with the medium and the latter boiled for a few minutes, the titration should be repeated as above described. If the desired reaction is not found to be present, then further adjustment by addition of the calculated amount of normal acid or alkali solution should be made. It is not to be expected that the first addition to the medium of the calculated amount of normal solution will give exactly the required reaction in every case. This is due to unknown side reactions which take place in the culture-media.

When the reaction has been sufficiently adjusted, the medium is to be filtered and is then ready to be distributed in test-tubes.

The methods of making the normal and twentieth normal solutions required may be found in standard works on chemistry. If one has not some knowledge of chemistry, he would better have the solutions made by a chemist.

The filling of the test-tubes with the fluid culture-media described in this section is best effected by means of a funnel of a capacity of about a liter. In this the fluid me-

dium is placed, and by means of a pinch-cock the requisite quantity of medium is run into each test-tube. In running the medium into the test-tubes the left hand holds the test-tube while the right hand removes the cotton stopper and manipulates the pinch-cock (Fig. 14). Care should be exercised not to allow any of the medium to come in contact with the neck of the test-tube, for it will make the cotton stopper

FIG. 14.—Method of filling test-tubes with culture-medium (Warren).

stick to the walls of the tube. To avoid this, the delivery-tube of the apparatus should be inserted some distance into the test-tube in filling.

The *quantity* of culture-medium run into each test-tube varies according to the form of culture desired and the character of the medium. In the case of liquid media and solid media designed to be used in the form of "stab" cultures the

tubes should be filled to a depth of 5 cm. For "slant" cultures of solid media a depth of about 3 cm. is sufficient, or enough to give a slanting surface from the bottom of the tube to about halfway up the opposite side.

Small *Ehrlenmeyer flasks* are sometimes used for bouillon cultures. These are of about 100 c.c. capacity, and are filled to a depth of about 1 cm. with the medium. The necks are provided with cotton stoppers, and the whole sterilized and treated as test-tube cultures.

STERILIZATION OF CULTURE-MEDIA.

In general, the sterilization of culture-media is effected by allowing them to remain exposed to the action of live steam in the steam sterilizer for twenty to forty-five minutes on three successive days. The period of exposure to live steam varies somewhat with the kind of culture-medium. A single exposure for the time mentioned is sufficient to destroy all bacteria present in what is called the vegetative or non-resistant form, but it will not kill *spores,* which represent a stage in the life-history of certain bacteria, in which form the organism is highly resistant to sterilizing agents.

Under favorable conditions, such as are to be found in culture-media at ordinary room-temperature, these spores develop into the vegetative or non-resistant form, which are easily destroyed by heat. Therefore, in order that the culture-medium be made sterile, it is necessary that it be again subjected to the action of steam on the following day for the same length of time, when the vegetative forms of the few surviving spores will have developed, and will be capable of destruction by ordinary exposure to live steam.

As a further precaution a third similar sterilization on the next day is necessary. Therefore, three steam sterilizations, of from twenty minutes to one hour each, on successive days, are required to keep culture-media sterile for an indefinite period.[1]

[1] As has been pointed out by Theobald Smith, this intermittent sterilization at 100° C. may not be sufficient in some cases to kill all the spores, because the condition in the media may not be favorable for their development into vegetative forms, between sterilizations. This seems to be especially true

A freshly prepared culture-medium must be sterilized on the same day that it is prepared, or by the next day it may be found to contain living bacteria, especially if kept over night in a warm room.

For the purpose of sterilization the test-tubes containing the media are to be placed in a round wire basket which fits into the steam sterilizer,[1] thus facilitating the handling of the tubes and also keeping them upright.

If the medium be in a flask ready for running into test-tubes, and if it be not convenient to do this the same day, the medium may be preserved as long as desired by inserting a cotton stopper into the mouth of the flask and then sterilizing as above indicated.

The time of each sterilization for bouillon, agar-agar, blood-serum, etc. may be fixed at half an hour; for potato-culture tubes and for litmus-milk, forty-five minutes.

In the case of gelatin, however, the time of exposure to live steam should be shorter, owing to the danger of destroying the solidifying power of the medium by too much heating. Twenty minutes' exposure is sufficient.

Large quantities of culture-media contained in flasks should be sterilized for forty-five minutes to an hour, for obvious reasons.

The sterilization of culture-media may also be effected in an **autoclave.** This is a steam-tight chamber for sterilizing by steam under pressure. Various forms of this apparatus are on the market. The great advantage of the use of this apparatus is that a single sterilization is sufficient. Exposure of culture-media in tubes, of glassware, and of other apparatus, in it to a temperature of 110° C. (6 lbs. pressure) for fifteen minutes suffices for sterilization in most cases. For the

of certain anaërobic spore-producing bacilli. Such spores may be the source of contamination of the culture-medium when it is placed under anaërobic conditions, or when the medium is used for anaërobic cultures, because strictly anaërobic bacteria may grow in company with other bacteria under aërobic conditions. Therefore, in those cases in which it is important to be certain of the absolute sterility of the culture-medium, sterilization in the autoclave (q. v.) is necessary.

[1] The "Arnold Steam Sterilizer" No. 5 is recommended.

sterilization of culture-media in bulk, about thirty minutes at this temperature is necessary.

In using the autoclave it is requisite that the confined air be replaced by superheated steam. To insure this, the time of sterilization should be reckoned only from the time when the theoretical temperature, as registered by the pressure-gauge, corresponds with that recorded by the thermometer.[1]

The Storage of Culture-media.—In order to prevent evaporation and the invasion of moulds, the cotton stopper should be cut off close to the mouth of the tube or flask, the surface of the stopper well singed with a flame, and the mouth of the tube or flask tightly closed with a cork.

Immediately before insertion, the portion of the cork that enters the tube or neck of the flask should be charred in a flame. If thought desirable, the cork may be sealed with paraffin.

II. BACTERIOLOGICAL EXAMINATIONS.

THE bacteriological examination of material obtained from the individual during life or at autopsies should determine whether bacteria are present or not, and if present their species and comparative number. At autopsies the examination should also determine the extent of the distribution of any infecting bacteria throughout the principal internal organs.

This is accomplished chiefly by means of two methods of examination—viz., the direct examination with the microscope of cover-glass preparations, and the results of cultures made from the tissues. Both of these methods should be employed together, but the culture method is perhaps the most important. A third but less frequent method is the inoculation of animals with pieces of tissue or material taken from the body.

Methods of Collecting Material.—In the bacteriological examination of pathological material obtained from the individual during life, it is of obvious importance that the

[1] Bacteriological Committee Report, *Jour. Amer. Pub. Health Assoc.*, Jan., 1898.

material be protected from the invasion of bacteria from without, and that in its collection every object with which it comes in contact be free from living bacteria.

To fulfil these requirements the material may be conveniently collected in any of the following ways:

1. It may be obtained directly from the individual by means of the sterilized platinum wire, and cover-glass preparations, cultures, and, if necessary, animal inoculations, made at once.

2. Since a very small quantity of the material usually suffices for the purposes of examination, it may often be very conveniently collected and brought to the laboratory on the so-called "swabs," where it can be subjected to the various manipulations at leisure.

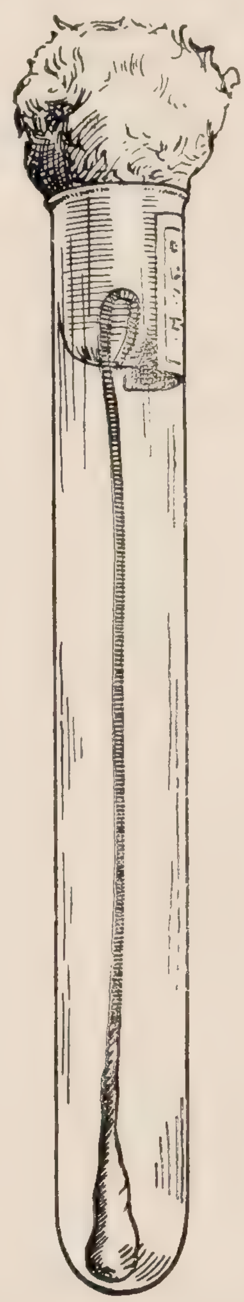


FIG. 15.—Sterilized test-tube and swab for collecting pus and fluids for bacteriological examinations (Warren).

The "swab" consists of a piece of rather stiff wire about six inches long, on one end of which is firmly twisted a pledget of absorbent cotton, so that the end of the wire is well covered. This is placed, cotton end first, in a test-tube, which is then provided with a cotton stopper (Fig. 15), and the whole sterilized in a hot-air sterilizer by heating to 150° to 180° C. during about half an hour. A large number of "swabs" in test-tubes may be kept on hand sterilized and ready for use.

When it is desired to secure material for bacteriological examination on a "swab," the cotton stopper is removed, the swab taken out, and the cotton end brought in contact with the pus or exudate in such a manner that some adheres to the cotton. The swab is then immediately replaced in the test-tube, the cotton stopper returned to its place, and the whole then carried to the laboratory.

In these manipulations care should be taken to avoid touching with the swab anything but the material which it is desired to examine, otherwise the material may be contaminated with other bacteria than those originally present in it.

By means of swabs material for examination from pus or exudates may be secured and brought to the laboratory in most instances. They are especially useful in surgical work, in which it is often desirable to determine the character of the organism present in a pus-formation or exudation without waiting to summon a bacteriologist or to collect the necessary cover-glasses, culture-tubes, platinum needle, etc. The swabs and their test-tubes may be kept on hand in a sterile condition, so that they may be handled by the operator or an assistant.

3. Fluid material may be collected by aspiration or otherwise. In the case of fluids care should be taken that everything with which the fluid comes in contact be clean and sterilized by heat if possible. The use of antiseptics, such as carbolic acid or corrosive sublimate, is to be avoided.

If a hypodermic syringe is used in obtaining material, it should be of a construction which will admit of sterilization by heat, and it should be so sterilized before using.

In the collection of pathological fluids, especially peritoneal exudates, a special form of apparatus has been found most useful. It consists essentially of a glass tube, about 14 cm. long and about 7 mm. in external diameter, one end of which is narrowed to a small opening and rounded off, while to the other end is attached a small rubber bulb like that on a "medicine dropper." It is to be kept ready for use in a test-tube, stoppered with cotton (see Fig. 16), the whole having been sterilized as are surgical dressings. The rubber bulbs are not expensive. Any number of pieces of this apparatus may be kept on hand in sterile condition.

When it is desired to obtain a sample of peritoneal or other fluid for bacteriological examination, the apparatus is removed from the test-tube and the fluid aspirated into it by

manipulation of the rubber bulb. It is then replaced in the test-tube. The fluid thus obtained should be free from contamination and may be readily transported to the laboratory for examination.

Cover-glass Preparations.—The method of demonstrating the presence of bacteria in pathological material by means of cover-glass preparations depends upon the fact that bacteria have the property of being colored by certain of the aniline dyes, and thus may be more readily seen by the microscope. The cover-glasses are best kept in alcohol, and as required for use wiped dry with a soft cotton cloth. A cover-glass preparation is made as follows: A very small amount of tissue or material to be examined is thinly spread over the surface of a clean thin cover-glass with the platinum wire or "loop" described on page 98 so as to give a streaked appearance to the surface, but not a definite layer which is ordinarily too thick for satisfactory examination. The charged cover-glass is then dried by holding it in the fingers over the flame of a Bunsen burner, and when dry it is placed, charged surface uppermost, in the grasp of a pair of cover-glass forceps,[1] by means of which it is passed rapidly three times through the flame of a Bunsen burner or alcohol lamp. This "fixes" the material on the glass, and the preparation is then ready for staining by one of the various methods given below. In staining, the cover-glass is held by means of the forceps with the charged side uppermost and level, and the surface is then completely covered

FIG. 16.—Apparatus for the collection of pathological fluids.

[1] The form of cover-glass forceps known as Stewart's is recommended They may be obtained from American dealers in bacteriological apparatus.

with the staining fluid, which is poured upon it from a dropping-bottle.[1] It may then be heated over the flame of the Bunsen burner, washed in water, and submitted to any further manipulation which may be necessary while still in the grasp of the forceps. When the staining is completed the preparation is next to be prepared for microscopic examination. This is done by placing the cover-glass, with as much water as will adhere to it, charged side downward, on a "slide," and then removing all remaining water, except a thin film of water between the slide and cover-glass, by gentle pressure with several thicknesses of filter-paper. The preparation is then ready for examination with an oil-immersion lens. The presence of this film of water is very essential for a satisfactory examination with the microscope, and its evaporation may be compensated for by a drop of water placed at the edge of the cover-glass. The preparation may also be mounted in balsam after carefully drying it first between filter-paper and then holding it in the fingers over the Bunsen flame, but the examination in water mount is better, because the apparent size of the bacteria is greater in this than when mounted in balsam.

It is of the greatest importance that cover-glass preparations be made from all exudates or acute inflammatory conditions of organs or tissues, so that the results of cultures can be controlled and some idea formed of the number and character of the bacteria present. They are of especial use as enabling one sometimes to recognize the presence of an organism which does not grow in the culture for some reason, and whose presence might escape notice, while their importance in many cases in the identification of the pneumococcus and the bacillus tuberculosis must be apparent. In certain cases it is best to examine the material in its natural state and without drying and staining. This is especially true of suspected infections with actinomyces and the amebæ coli. In these cases the material should be spread over a cover-glass, and this placed, while the material is still

[1] The form of drop-bottle known by dealers in bacteriological supplies as the "T. K. patent," with flat stopper, is the best.

moist, charged surface downward, on a "slide," and then examined with various powers of the microscope. If necessary, a small amount of normal salt solution (0.6 per cent.) may be added to dilute the material and facilitate the examination.

Staining Methods for Cover-glass Preparations.[1]—**Simple staining** is used for the demonstration of bacteria in general, and also useful in gaining an idea of the character of the cellular elements in the preparation.

Löffler's alkaline methylene-blue solution is perhaps the best staining fluid to use for simple staining, for it does not stain so diffusely and intensely as do the other commonly used dyes, such as fuchsin and gentian violet, which may also be employed.

The cover-glass, covered with the staining fluid, should be warmed over the Bunsen flame, so that the fluid steams, for about fifteen seconds. Boiling should be avoided. The preparation is then washed in water for two or three seconds and mounted.

Pappenheim's pyronin and methyl-green mixture may be used as above described in place of Löffler's methylene-blue solution. This mixture is composed of:

Saturated aqueous solution of methyl-green,	3–4	parts;
" " " " pyronin,	1–1½	"

Bacteria are stained brilliant red and the nuclei of cells are stained blue or purple. The mixture is said to keep several weeks.

Gram's Method of Staining.—1. Cover the preparation with aniline-gentian-violet solution for thirty seconds.

2. Wash in water for two or three seconds.

3. Cover the preparation with Gram's solution of iodin for thirty seconds.

4. Wash with 95 per cent. alcohol until the color ceases to come out of the preparation.

5. Wash in water for two or three seconds and mount.

Certain bacteria are stained by this method, while others are not. Bacteria when stained by it appear dark blue or

[1] The formulæ for staining fluids will be found in Part III. (see Index).

black, while the nuclei of the cells are rather faintly stained or not stained at all. The method is especially useful in the demonstration of bacteria which are stained by it when they are present in small numbers or when a few Gram-staining bacteria are mixed among numbers of bacteria which do not stain by this method. It also has some value as a means of differentiating between bacteria which may be very much alike in size and shape.

In the following table the behavior of the more important pathogenic bacteria toward the method of Gram is indicated:

Stained by Gram's Method.	Decolorized by Gram's Method.
Staphylococcus pyogenes aureus.	Gonococcus.
Staphylococcus pyogenes albus.	Diplococcus intracellularis meningitidis.
Streptococcus pyogenes.	Typhoid bacillus.
Streptococcus capsulatus.	Bacillus coli communis.
Pneumococcus.	Spirillum of Asiatic cholera.
Micrococcus tetragenus.	Bacillus pyocyaneus.
Bacillus diphtheriæ.	Bacillus of influenza.
Bacillus tuberculosis.	Bacillus of glanders.
Bacillus of anthrax.	Bacillus proteus.
Bacillus of tetanus.	Bacillus mucosus capsulatus.
Bacillus aërogenes capsulatus.	Bacillus of dysentery.
Bacillus of malignant edema.	Bacillus of bubonic plague.
	Bacillus of chancroid.

W. H. Smith's Method of Staining Capsulated Bacteria in Body Fluids.—1. Make a thin cover-glass preparation from fresh sputum, or pneumonic, pleural, or pericardial exudate.

2. Pass through flame.

3. Cover with a 10 per cent. aqueous solution of phosphomolybdic acid (Merck) four to five seconds.

4. Wash in water.

If the micro-organism is Gram-staining, like the pneumococcus or streptococcus capsulatus, proceed as follows:

5. Aniline oil gentian violet, steaming one-quarter to one-half minute.

6. Wash in water.

7. Treat with Gram's solution of iodin, steaming one-quarter to one-half minute.

8. Decolorize with 95 per cent. alcohol.

9. Wash in water.

10. Stain with a 6 per cent. aqueous solution of eosin (Grübler's eosin, w. g.) one-half to one minute, warming gently.

11. Wash in water.

12. Wash in absolute alcohol.

13. Clear in xylol and mount in xylol balsam.

The capsule will be found to be distinct, clear cut, eosin-stained about the Gram-stained micro-organism.

If the micro-organism is Gram-decolorizing, after step 4 above proceed as follows:

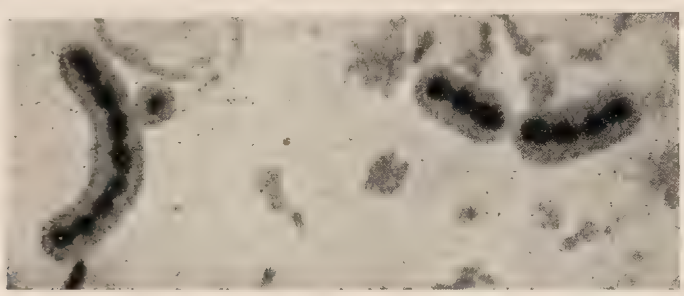


FIG. 17.—Capsulated micrococci in a cover-glass preparation from an endocarditic vegetation stained by W. H. Smith's method; × 2000 (W. H. Smith; photo. by L. S. Brown).

5. Stain with a 6 per cent. aqueous solution of eosin one-half to one minute, warming gently.

6. Wash in water.

7. Counterstain with Löffler's methylene-blue solution one-quarter to one-half minute, warming gently.

8. Wash in absolute alcohol.

9. Clear in xylol and mount in xylol balsam. The capsule will appear eosin-stained about the blue-stained micro-organism.

Special staining methods for cover-glass preparations will be found in the section on Special Bacteriology.

Examination by Cultures.—The demonstration of the presence of bacteria in a tissue or exudate by means of cultures consists in bringing a small amount of the material to be examined in contact with some solid nutrient substance in which the bacteria will thrive. On this the bacteria by multiplication form masses or colonies visible to the naked eye, and present appearances which enable a practised eye in many cases to recognize the species of the bacteria of

which they are composed. Of the solid culture-media described in the preceding section, the *coagulated blood-serum* is distinctly the best to use for the demonstration of the presence of bacteria in routine pathological work, because certain of the most important pathogenic bacteria grow better upon it than upon agar-agar or similar media. The other media have important uses in the study of the bacteria after their isolation from the tissues, and in certain instances special media are to be used, as will be pointed out in the following pages. The blood-serum medium here described has been found entirely suitable for the isolation of the bacillus tuberculosis from tubercular lesions, which proves its efficiency as a culture-medium.

Method of Preparing Cultures on Blood-serum.—The preparation of cultures on the coagulated blood-serum consists in distributing over the surface of the medium in a test-tube as much of the tissue or other material as will adhere to the end of a piece of stiff platinum wire hammered flat at the end. The wire is fixed in the end of a glass or metal rod, and should be about 8 cm. long. It should have a rounded spatula-like extremity, and should be thick enough not to bend easily. In making cultures from clinical material, the platinum loop may be used for fluids.

The "platinum wire" or "loop" consists of a piece of platinum wire of about 22 gauge, 2½ to 3 inches long, fixed in the end of a small glass or metal rod 8 or 10 inches long. It is often of great convenience to have two of these instruments, one with the wire curled into a simple loop about 1 to 2 mm. in diameter at the free end, and the other a straight wire with the free extremity hammered flat into a very small spatula. The latter is of great utility in picking up minute portions of bacterial colonies.

Both this instrument and the stiffer wire, above mentioned, should be heated to a red heat in a flame immediately before using, in order to destroy any bacteria that may be upon them.

If the material is on a "swab," the surface of the blood-serum or other media may be conveniently inoculated directly

by gently rubbing the swab over it. In this case it is usually best to make a dilution or two by means of the platinum wire, as described below, especially if there be a large amount of material on the swab or if the cover-glass examination has shown that a large number of bacteria are present. In any case it is important that the infected material be spread over all of the surface of the medium, and not in the form of one or two narrow streaks.

It is, of course, essential that the material brought in contact with the culture-medium should be free from bacteria not originally present in it, or that it be not contaminated with bacteria from outside sources. Therefore, in taking material from the interior of organs and tissues the surface is first sterilized by searing it with a hot knife, such as an ordinary case-knife, which has been heated in the Bunsen flame, and then, through a small incision made with another hot knife in this seared or sterilized area, the material from the interior is collected on the end of the platinum wire, which has also been previously heated in the Bunsen flame to sterilize it, and then cooled either by plunging it in the water of condensation of the culture-tube for a few seconds or by moving it about in the interior of the tissue.

In the case of exudations on free surfaces, however, this searing is impossible, and therefore care should be exercised at the autopsy not to contaminate any such exudate by handling before the material for culture has been obtained with the platinum wire. The material thus secured is then transferred by means of the platinum wire to the surface of a blood-serum culture-tube, and the infected wire gently rubbed over *all* of the surface of the culture-medium, avoiding, however, the breaking of the surface. *It is important that the material be well distributed over the nutrient surface.* If the material is suspected of containing a large number of bacteria, as in the case of suppurations or acute inflammatory lesions, a second tube should be inoculated from the first one by touching the platinum wire, previously sterilized and cooled, to the infected surface of the first tube, and then gently rubbing the infected wire over the surface of the

second tube. This operation is called "diluting." The object of this is to obtain, after the development of the culture, a sufficiently small number of colonies in the second tube, so that they may be discrete—*i. e.*, separated from one another—and thus be enabled to exhibit their characteristic appearances, which are largely lost when the colonies are so numerous as to be confluent.

If thought desirable, a third tube may be similarly inoculated from the second, but this is rarely necessary. In making these "dilutions" it is well to cool the platinum wire in the water of condensation of the sterile tube before touching it to the infected surface of the other tube. As a rule, one tube will be sufficient to obtain discrete colonies from organs or tissues in which no suppurative or exudative condition is present.

Anaërobic cultures are indicated in certain cases. For anaërobic methods, see pp. 123, 124.

After the manner above indicated cultures are to be made at the autopsy as a matter of routine from the *blood of the heart*, from the *liver*, the *spleen*, the *lung*, and the *kidney*. Cultures are also to be made from any acute inflammatory lesion in any situation.

As each culture-tube is infected it is to be labelled with the name of the organ or of the material from which it was infected, and with the date. For this purpose small paper labels coated with mucilage are used.

The culture from the blood of the heart should be made before the removal of that organ from the body, by searing the right ventricle and then puncturing it with a sterilized knife to admit the platinum wire. The amount of blood used for the culture should be as much as will adhere to the platinum wire. Cultures from vegetations in acute endocarditis are not usually of much value unless they are sufficiently large to enable a sterilization of their surface to be effected and material for culture secured from their interior.

Cultures from the Blood during Life.—This procedure is coming more and more into use for diagnostic purposes in cases of suspected bacterial infection of the

circulating blood. The blood is obtained from one of the large veins at the flexure of the elbow by means of an antitoxin or similar syringe, and in most cases is immediately mixed with an excess of sterile bouillon. About 10 c.c. or more of blood should be taken. A convenient way is to distribute the blood directly by means of the syringe in quantities of 3 or 5 c.c. among flasks, each containing 200 to 400 c.c. of sterile bouillon. The blood is thus highly diluted in order to obviate its bactericidal action.

The addition of sterile ascitic fluid to the bouillon in the proportion of 1 part to 3 or 4 parts of bouillon is desirable in cases in which streptococcus or pneumococcus is suspected.

In the case of general infection with anaërobic bacteria the blood should be mixed immediately with melted agar-agar at 40° C. in test-tubes, in the proportion of 1 part to 2 or 3 parts of agar-agar. The mixture should fill the culture-tubes to a height of about 8 cm. For the method of cultivating gonococci from the blood see page 147.

The strictest aseptic precautions must be observed in obtaining the blood and in mixing it with the culture-medium.

Intraperitoneal inoculation of mice may also be made with ½ to 1 c.c. of the blood. This may give rise to streptococcus or pneumococcus septicemia.

McJunkin's Tube for Collecting Blood for Cultures.—The blood is drawn from a vein, as for the Wassermann reaction, directly into a large test-tube containing an oxalate solution, which prevents clotting, but does not inhibit the growth of any organisms present. The blood is perfectly protected from contamination, so that it can be carried to the laboratory, where it can be inoculated into media in tubes and flasks, and, in addition, be plated. The method simplifies the technique of obtaining blood-cultures by eliminating the necessity of carrying much apparatus to the bedside, where inoculations are always made under difficulties, and by rendering it easily possible to take blood-cultures from several patients on one trip.

The preparation of the tube is simple. In a test-tube (*a*) which measures 20 by 200 mm. (Bausch and Lomb, No.

16,920) there are placed 15 c.cm. of a solution which contains 2 grams of ammonium oxalate and 6 grams of sodium chlorid to the liter of distilled water. This is marked (*b*) in the figure. Cotton 4 cm. wide (*c*) is now wrapped around

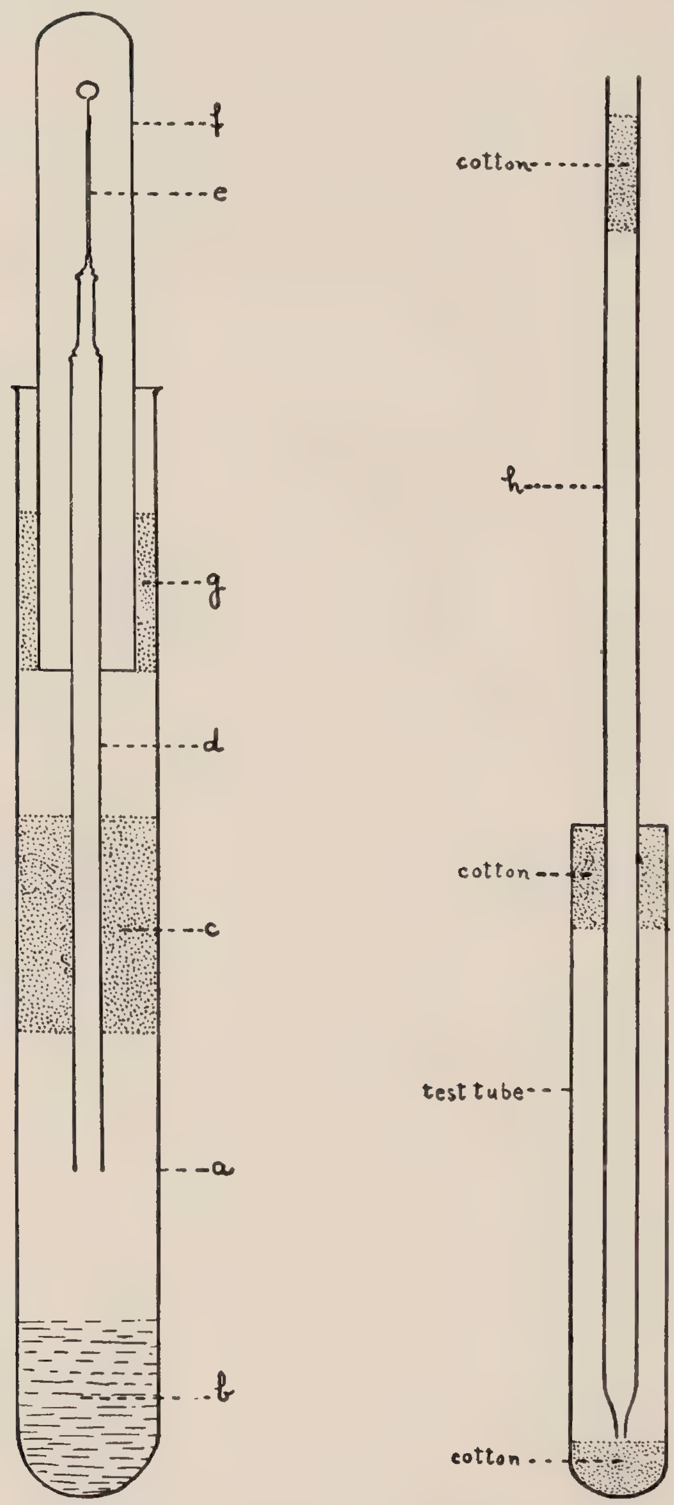


FIG. 18.—McJunkin's tube for collecting blood for cultures.

the rubber tubing (*a*), which is 150 mm. long, with a 1-mm. wall and a 3-mm. lumen, and into the upper end of this tubing there is inserted a 19-gauge needle 1 inch long (*e*). The needle is capped by inserting into the upper end of the large tube a smaller one (*f*), which measures 12 by 100

mm. (Bausch and Lomb, No. 16,920), and which has wrapped around the lower end a 3-cm. plug of cotton (*g*). Both cotton plugs should fit snugly. The tube complete is autoclaved for twenty minutes at 110° C. The needles should be fitted with as large a stylet as possible, so as to insure patency of the needle.

After the needle has been inserted into the vein, a few seconds are required for the blood to pass through the tubing. The lower cotton plug eliminates all chances of contamination, and when it is removed in the laboratory the upper end of the tube is flamed and the blood removed with a 10-c.cm. sterile pipet (*h*). The tubing and needle are cleansed by forcing through them a few cubic centimeters of water from a syringe, after which they are placed in a saturated solution of borax.

The Inoculation of Animals.—The inoculation of animals directly with pathological material is often of important diagnostic value. In routine pathological work it is mainly useful in determining the presence of the pneumococcus or of the bacillus tuberculosis when the ordinary methods are considered inadequate.

The methods of inoculating animals are described on pp. 118–123.

III. THE METHODS OF STUDYING BACTERIA IN CULTURES.

THE cultures made as described in the last section, having been in the incubator for eighteen to twenty-four hours, are next to be studied, and the identity or diagnosis of the bacteria whose colonies have grown out upon them is to be established. The identification of the infecting bacteria present in most cases may be made from a consideration of the size, color, and general appearance of the colonies as they appear on the surface of the blood-serum when taken in connection with the morphology of the bacteria composing them. In some cases, however, this may not be sufficient evidence upon which to base the diagnosis,

and it may be necessary to obtain further facts in regard to a given organism in order to identify it with a sufficient degree of certainty. Thus it may be necessary to observe the appearances of its growth in pure culture in various media, and to ascertain whether it produces certain chemical changes in the media by its growth. Its ability to grow with or without oxygen, its reaction toward staining agents, whether it has independent motion or not, and its effects upon animals by inoculation, are also points which may have to be determined to enable one to make a positive diagnosis of the species to which the organism belongs. Therefore, in the study of the colonies of the bacteria which have developed in the cultures a familiarity with certain fundamental bacteriological methods is necessary. These will be described in this section.

1. COVER-GLASS PREPARATIONS FROM CULTURES.

A minute proportion of a colony or bacterial growth, the component organisms of which are to be examined, is picked up on the end of the platinum wire, which has been previously heated in the Bunsen flame and cooled, and is thinly distributed on the surface of a cover-glass by gentle movements of the platinum wire. It is very important that the bacteria should be more or less separated from one another in places, so that a good view of the individual organisms may be obtained. This can often best be effected by placing a minute drop of water on the cover-glass first, and then moving the infected end of the platinum wire back and forth through this. The preparation is then dried between the fingers over the Bunsen flame, and next, having been grasped with the Cornet forceps, is to be rapidly passed three times through the Bunsen flame. While still held by the forceps, it is then stained by covering it from a dropping-bottle with the staining solution, and washed in water or submitted to any other manipulation which may be required.

The staining solutions ordinarily employed are carbolic-fuchsin, aniline-gentian-violet, and Löffler's alkaline methylene-blue. If methylene-blue be used, it is best to steam

the preparation in staining for about ten seconds, but in the case of the other stains this is not necessary, for they stain deeply almost immediately, so that the staining solution need not remain longer in contact with the bacteria than a few seconds. After staining, the cover-glass is to be washed in water and mounted for examination, as described on page 92 in the case of cover-glass preparations from tissues.

Gram's method of staining may also be used for cover-glass preparations from cultures. As has been pointed out elsewhere (see page 95), this method does not stain all species of bacteria, but some species are stained by it and others are not. This fact is sometimes useful in aiding in the identification of a given organism, and as enabling one to recognize the presence of a few Gram-staining bacteria among a large number of others which are unstained.

In this connection it should be pointed out that bacteria which are stained by this method, when taken from cultures a few days old, may not be stained by it if taken from older cultures. Therefore, Gram's method, if used as a means of differentiation, should be applied only to bacteria in actively growing cultures.

Bacteria stained by this method have a blue-black color.

For a list of bacteria that stain by Gram's method see page 96.

The Staining of Spores.—Spores take up the anilin dyes with difficulty, probably owing to their dense protective envelope. When once stained, however, they do not give up their color easily, and resist decolorizing agents. The cover-glass preparations should be thinly spread.

Abbott's Method.—1. Stain the cover-glass preparation deeply with methylene-blue, heating repeatedly until the staining solution boils, but do not boil continuously, during about one minute.

2. Wash in water.

3. Wash in 95 per cent. alcohol containing 0.2 to 0.3 per cent. hydrochloric acid.

4. Wash in water.

5. Stain for eight to ten seconds in aniline-fuchsin solution.

6. Wash in water and mount.

The spores are stained blue and the bodies of the bacteria red.

Moeller's Method.—1. Wash the cover-glass preparation in chloroform for two minutes.

2. Wash in water.

3. Treat with 5 per cent. solution of chromic acid one-half to two minutes.

4. Wash in water.

5. Stain with carbol-fuchsin, heating slowly until the fluid boils.

6. Decolorize well in a 5 per cent. solution of sulphuric acid.

7. Wash in water.

8. Stain in aqueous solution of methylene-blue (1 gram to 100 c.c.) thirty seconds. The spores will be red, the bodies of the bacteria blue.

The preliminary treatment with chloroform is to cleanse the preparation.

Fiocca suggests the following rapid method: "About 20 c.c. of a 10 per cent. solution of ammonia are poured into a watch-glass, and ten to twenty drops of a saturated aqueous solution of gentian-violet, fuchsin, methylene-blue, or safranin added. The solution is warmed until vapor begins to rise, then is ready for use. A very thinly-spread cover-glass, carefully dried and fixed, is immersed for three to five minutes (sometimes ten to twenty minutes), washed in water, washed momentarily in a 20 per cent. solution of nitric or sulphuric acid, washed again in water, then counterstained with a watery solution of vesuvin, chrysoidin, methylene-blue, malachite-green, or safranin, according to the color of the preceding stain. This whole process is said to take only from eight to ten minutes, and to give remarkably clear and beautiful pictures."

The Staining of Flagella.—All motile bacteria are provided with delicate wavy, hair-like prolongations of their protoplasm, called flagella, which are of comparatively great length. These flagella are the locomotor organs of the organism. The number of them attached to each individual varies to a considerable extent with the species of the bacteria. Thus the individuals of some species have but one flagellum, while the individuals of other species may have few or many springing from all parts of the organism.

The flagella are not rendered visible by the ordinary methods of staining, but special methods are necessary for their demonstration. These methods depend essentially upon the use of a mordant, which causes the flagella to take up the stain.

The cover-glasses must be absolutely free from grease in these methods, so that the watery fluids may be spread evenly over them and not run into patches. The cover-glasses may be prepared by warming them in concentrated sulphuric acid for a time, washing them in water, and keeping them in a mixture of equal parts of alcohol and strong ammonium hydroxid solution.

When used they are to be dried on a cloth which has previously been soaked in ether and allowed to dry, in order that it may contain no trace of fat. Another way to treat the

cover-glasses is to take them from alcohol, dry them with a clean cloth, and then heat them by means of the cover-glass forceps in the Bunsen flame to burn off any fat or grease.

The bacteria must be distributed upon the cover-glass well separated from one another in these methods. They should not be subjected to too much manipulation in doing this, for the flagella are readily broken off. A good way is to make a dilute suspension of the bacteria in distilled water, and place one or two loopfuls of this on the cover-glass, not spreading with the loop, but making the suspension flow over the surface by inclining the cover-glass.

Another way is to place two drops of water on a cover-glass—to draw the infected wire once through one of them across the surface, and then once through the other drop, thus making two streaks. This subjects the bacteria to less manipulation and gives a good distribution in places.

The cover-glasses prepared as above indicated are to be allowed to dry in the air, and are then to be heated for a few seconds over a flame while held between the fingers. They are then ready to be stained by any of the methods given below. The cultures used for the preparations should not be older than eighteen to twenty-four hours. Solid culture-media, such as agar-agar, should be employed.

Löffler's Method.—Treat the preparation for about one minute with the freshly filtered mordant solution, which is—

Aqueous solution of tannic acid (20 grams tannic acid to 100 c.c. water),	10 c.c.;
Cold saturated solution of ferrous sulphate,	5 c.c.;
Saturated aqueous or alcoholic solution of gentian-violet or fuchsin,	1 c.c.

The cover-glass is to be covered with this while held with the cover-glass forceps, as in ordinary methods of staining. The mordant, thus placed on the cover-glass, may be gently heated by holding the preparation high over the flame for a period of about one minute, but it must not be boiled. After this the preparation is to be washed in water, and then stained with a freshly prepared and filtered solution of aniline-gentian-violet or aniline-fuchsin, with gentle heating for

thirty to sixty seconds. It is then again washed in water, and mounted in water or balsam for examination.

In using this method, as well as others, an important thing to avoid is overheating. The mordant may be freshly mixed every time or kept indefinitely for use.

The ferrous sulphate solution should always be freshly prepared, for it rapidly decomposes. The solution of tannic acid keeps well, however.

The addition of varying quantities of acids or alkalies for different species of bacteria, as recommended by Löffler, is not necessary.

Pitfield's Method as Modified by J. Blackburn Smith.—The mordant is prepared as follows:

A saturated solution of mercuric chlorid, made by boiling, is poured while still hot into a bottle in which crystals of ammonia alum have been placed in quantity more than sufficient to saturate the fluid. The bottle is then well shaken and allowed to cool. To 10 c.c. of this fluid 10 c.c. of a freshly made 10 per cent. solution of tannic acid are added and 5 c.c. of carbol fuchsin solution. After mixing, filter. This mordant will keep.

In staining, the mordant is filtered on to the cover-glass preparation, which is heated until steam is given off during about three minutes. Boiling is to be avoided. The preparation is then washed in distilled water and is stained in a mixture of 1 c.c. of a saturated alcoholic solution of gentian violet and 10 c.c. of a saturated solution of ammonia alum. This mixture is filtered on to the preparation.

Bowhill's Method.—Stain the preparation in the following solution for ten to fifteen minutes, slightly warming:

Saturated alcoholic solution of orcein,	15 c.c.
Aqueous solution of tannin, 20 : 80,	10 c.c.
Distilled water,	30 c.c.

This mixture is to be filtered before using. The saturated alcoholic solution of orcein should be at least ten days old.

G. H. Neuman's Method.—Make this mixture:

Tannic acid,	1 gram;
Potassium alum,	1 "
Distilled water,	40 c.c.

Add to this, after the ingredients are dissolved, 0.5 gram of night blue, dissolved in 20 c.c. of absolute alcohol, mixing thoroughly. Filter off the precipitate that forms. The filtrate is the staining fluid. It will keep only a few days and should be filtered immediately before use.

1. Stain the preparation (which has been dried at room-temperature) for two minutes or more, changing the fluid two or three times.

2. Wash thoroughly in water.

3. Stain with a saturated aqueous solution of gentian-violet for two minutes, in order to stain the bodies of the bacteria.

4. Wash in water and mount.

Williams' Method.—This is a modification of van Ermengem's method along the lines of the modification of Hinterberger and others. It has been adopted by Dr. Hugh Williams after a large experience with various methods in the Laboratory of the Massachusetts General Hospital.

The method is capable of giving black bacteria and flagella, with little or no precipitate. The method is as follows:

1. Cover the cover-glass with a mordant consisting of

Alumnol,[1] 1 per cent. solution,	5 c.c.;
Osmic acid, 2 per cent. solution,	5 c.c.;
Tannin, 20 per cent. solution,	15 c.c.

Shake the mixture, and add three drops of glacial acetic acid, and again shake.

2. Apply the mordant less than one minute without heating. Wash thoroughly in water.

3. Cover the preparation, during about one minute, with a 1 per cent. solution of silver nitrate to which sufficient ammonium hydroxid has been added to keep the silver in solution.

4. Wash in water.

5. Wash with 0.6 per cent. solution of sodium chlorid.

6. Flood the preparation with a 30 per cent. solution of ammonium hydroxid, and immediately wash in water.

7. Apply a few drops of Ortol photographic developer.

[1] Farbwerke vorm. Meister Lucius u. Brüning, Höchst a. M., Germany.

The directions for making up this developer come with the Ortol.

8. Wash in water.

9. Cover with a 1 per cent. solution of gold chlorid during a few seconds.

10. Wash in water, and apply Ortol developer for a few seconds.

11. Wash in water, and cover with a 1 per cent. solution of mercuric chlorid for a few seconds.

12. Wash in water.

13. Apply Ortol developer for a few seconds.

14. Wash in water, and repeat the application of chlorid of gold, the washing, and the application of the developer two or more times. Between the various applications of the chlorid of gold the preparation should be inspected with a high, dry lens to determine the progress of the staining. This is readily done by placing the cover-glass, charged side upward, on a slide. In this way the process of impregnation with gold may be controlled; for the flagella, if stained, may be easily seen with the high-power dry lens.

The preparation is very conveniently held during the process in cover-glass forceps. The washing is best done in a small stream of water from a faucet. The various solutions are conveniently applied from dropping-bottles, see p. 94.

It will be seen that the process consists essentially in the impregnation of the flagella with silver, followed by intensification, in the photographic sense, with mercury and gold. The object of the application of the sodium chlorid and ammonia is to remove the excess of silver compounds which adhere to the surface of the cover-glass in spite of washing. This excess of silver compounds is chiefly responsible for the precipitates which appear on the preparation after the intensification. In spite of the application of the sodium chlorid and ammonia solutions, some precipitate will occur if the intensification is pushed too far. On this account it is advisable to observe the progress of the intensification under the microscope as above indicated.

Although this method may appear complicated, in practice it requires but a few minutes to stain a preparation.

2. METHODS OF OBTAINING PURE CULTURES.

When it is desired to obtain a pure culture of bacteria, a colony or a portion of a colony of the organism is secured on the end of the sterile platinum wire, and transferred by this means to the culture-medium in another test-tube. The bacteria thus sown in the fresh culture-medium multiply there, and produce a growth visible to the naked eye which exhibits appearances more or less characteristic of the species. This growth, if the medium be a solid one, will usually be in the form of confluent colonies; if the medium be a fluid one, the growth may appear as a sediment with or without clouding of the liquid, or it may manifest other peculiarities according to the species to which the organism belongs. If other bacteria are present in the culture from which it is desired to obtain material for a pure culture, it is important that the material should be taken from a colony of the organisms which is well separated from other colonies —*i. e.* that the colony should be a so-called "discrete" one.

In transplanting, the culture-tube containing the colony and the culture-tube that is to be infected from it are held side by side in the left hand in a slanting position in such a way as to give a good view to the operator of the surface of the media in each, while the cotton stoppers are removed and held between the fingers of the same hand (Fig. 19). The object of holding the tubes in a slanting position is to offer less chance of contamination from bacteria gaining entrance to the culture-medium from the air.

The platinum wire, which is manipulated by the right hand, is first sterilized by holding in the Bunsen flame until it glows, and then cooled by contact with the media to be infected, after which its free end is carefully brought in contact with the discrete colony or pure culture-growth, and immediately inserted into the sterile tube to inoculate it. The manner of inoculating the sterile culture-medium in the other tube with the infected platinum wire will vary with the form and character of the culture desired.

If the medium to be inoculated is a fluid one, the wire is simply immersed in it and moved back and forth once or

twice. If the medium be a solid one in the form of a slant, the infected end of the wire is drawn over the surface once or twice from the bottom of the slant to its upper end; or if the solid medium in the tube be arranged for a stab culture (see page 78), the infected wire is to be plunged once through the center of the mass to the bottom of the tube. After the tubes have been inoculated as above indicated, the wire is to be immediately withdrawn and the cotton stoppers replaced. They are then to be placed in the incubator for development. Gelatin cultures, however, must not be so treated, but are to be kept at room-temperature, for the heat of the incubator would cause the gelatin to become fluid.

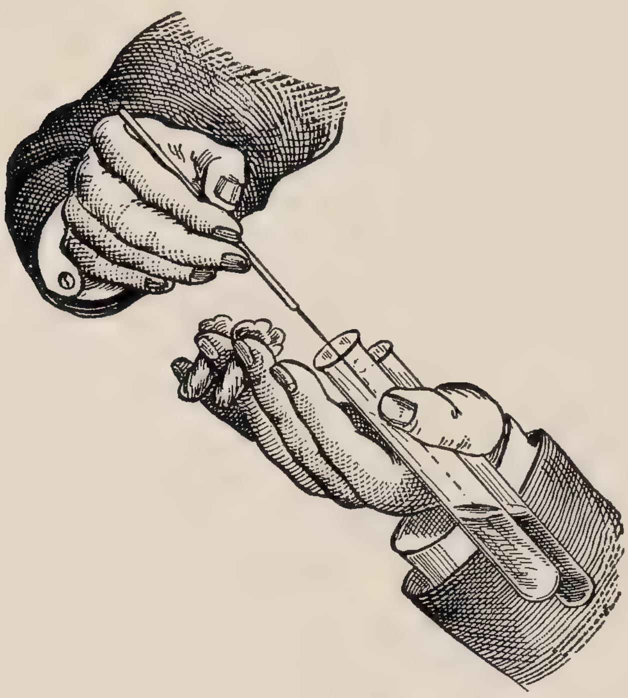


FIG. 19.—Method of holding tubes during inoculation.

These details as to the manner of manipulating the culture-tubes, cotton stoppers, and platinum wire also apply to the procedure described below.

Method of Isolation of a Bacterium in Pure Culture from a Mixed Growth.—If there is a more or less confluent growth of colonies of various kinds in a culture-tube, and it is desired to isolate a pure culture of one of the species of bacteria present, it is obvious that the first step is to obtain separate or "discrete" colonies of that organism. This is accomplished by securing a minute quantity of the growth on the end of the sterilized platinum wire (preferably from a spot where the organism is prevalent), and distributing this over the surface of a sterile blood-serum tube by gently rubbing the end of the infected wire as thoroughly as possible over it. The wire is then sterilized in the Bunsen flame, cooled in the water of condensation of a second sterile blood-serum tube, next touched to the infected surface of the first tube, and the wire thus infected gently and thoroughly

rubbed over the surface of the second. In a similar manner a third tube is then infected from the second, and then all the tubes placed in the incubator for eighteen to twenty-four hours. It is evident that comparatively few bacteria will be sown on the medium of the second tube, and still fewer on that of the third, so that the number of colonies which develop in the second tube will be less numerous than in the first tube, and those in the third tube still smaller in number. Therefore, in either the second or the third tube, or in both, the bacteria sown may be sufficiently few for discrete colonies to develop from them, and among these there may be some composed of the bacterium which it is desired to isolate. From one of such discrete colonies pure cultures may then

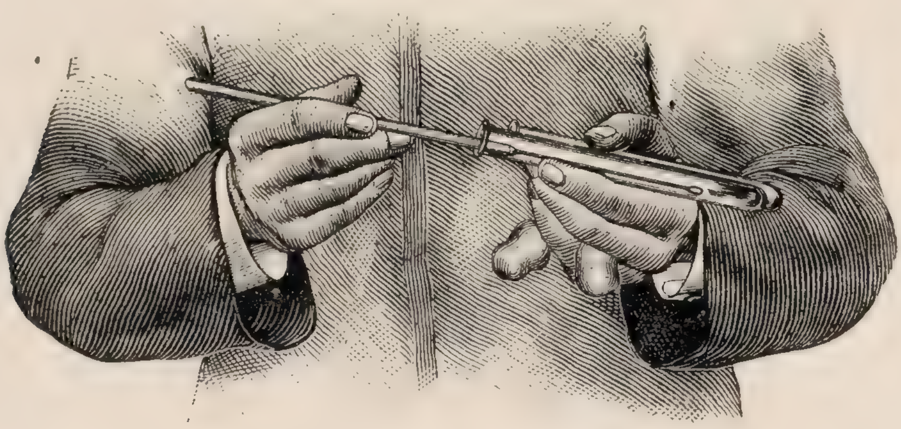


FIG. 20.—Diluting cultures.

be prepared as described above. The second and third tubes used in this method are called "dilutions." The details of the manner of manipulating the tubes, etc. in this method may be understood from the description given on page 111 and from Fig. 20.

The Plate Method of Petri.—Another method for obtaining discrete colonies of an organism from a mixed growth of several species is that known as the plate method of Petri. This is a modification of the original complicated method of Koch.

The method consists in making "dilutions" in melted agar-agar or gelatin tubes, and then pouring the infected medium into shallow glass dishes (Fig. 21) previously sterilized, in which it is allowed to solidify. A few bacteria are thus distributed throughout a thin layer of culture-medium in the "dilutions," and the colonies which develop from them are then more or less separated from one another, so that

8

pure cultures may be obtained from them. In carrying out this method the procedure is as follows:

Three sterile gelatin or agar-agar tubes are melted by heat and placed in a water-bath warmed to between 40° and 42° C. for several minutes, to bring the culture-medium to this temperature. This temperature is important especially in the case of agar-agar, for it is just above the solidifying point of that medium (38° C.) and yet not injurious to the vitality of

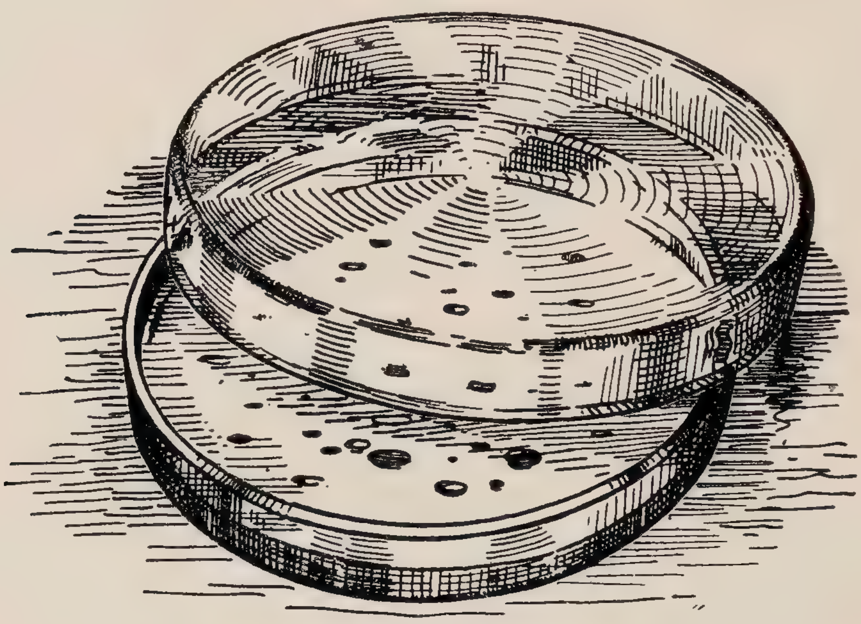


FIG. 21.—Petri dish with colonies.

the bacteria. The tubes are then infected successively from the bacterial growth or from the pathological material from which it is desired to obtain discrete colonies, in the same manner as described for the method with blood-serum tubes —viz. one tube being inoculated from the growth or tissue, a second tube or dilution from the first tube, and a third tube or dilution from the second tube, the platinum wire being sterilized after each inoculation. For making the "dilutions" a platinum wire bent into the form of a small loop (see page 98) is to be used, and as much of the culture-fluid as will adhere to it used for inoculating. The wire should be moved back and forth several times in the medium of each tube when inoculating it, in order to ensure a good distribution of the bacteria throughout the fluid. The contents of each tube thus inoculated are then poured into sterilized Petri dishes, in which the culture-medium solidifies in a thin layer.

The Petri dishes (Fig. 21) are of clear glass, circular in form, 10 cm. in diameter and about 1 cm. deep. Each is

provided with a loosely fitting flat cover of glass. These dishes with their covers are to be sterilized before using by placing them in the steam sterilizer for half an hour or by heating them to 150° C. in the hot-air sterilizer. When cool they are ready to receive the contents of the inoculated test-tubes. In pouring, the cover of the dish is not to be removed any more than is necessary, and it is to be immediately replaced, so that contamination from the air may be better avoided. It is very desirable that there be no dust about the place where the dishes are "poured," and no currents of air.

If agar-agar is used, the dishes thus prepared are to be put in the incubator for eighteen to twenty-four hours as soon as the medium is solid, which it becomes in a few minutes; but if gelatin be used, the dishes are to be set aside in a cool place, free from dust, to solidify, and are then to be kept at room-temperature for several days. Colonies first begin to appear in the gelatin usually after forty-eight hours.

The method of Petri is of great utility in the study of bacteria from the botanical standpoint, for it is especially adapted for the study of the appearances of colonies under the low power of the microscope. It is, however, inferior to the method with blood-serum tubes for routine pathological work, for the following reasons: First: Certain pathogenic bacteria grow only feebly on the culture-media which it is necessary to employ in this method, while they grow comparatively vigorously on blood-serum. Second: The method is complicated and much more troublesome and time-consuming than the simple method described above.

The Determination of the Motility of Bacteria.— This is done by observing the individual organisms, unstained, in a drop of bouillon or similar fluid under the oil-immersion lens. For this purpose a so-called "*hanging drop*" is prepared, for which a special form of slide known as a "*hollow slide*" is necessary. The hollow slide is a slide having a shallow circular concavity, about 1 cm. in diameter, ground out in its center (Fig. 22).

In **preparing a hanging drop** the procedure is as follows: A small drop of a bouillon culture or of the water of con-

densation of a blood-serum or agar-agar slant is placed in the center of a cover-glass by means of the platinum wire. The cover-glass is then placed, drop downward, over the circular depression in the hollow slide. To hold the cover-glass in its place and to prevent evaporation of the fluid in which the organisms are suspended, a little vaselin is painted around the margin of the depression before placing the cover-glass in position. The hanging drop thus prepared is

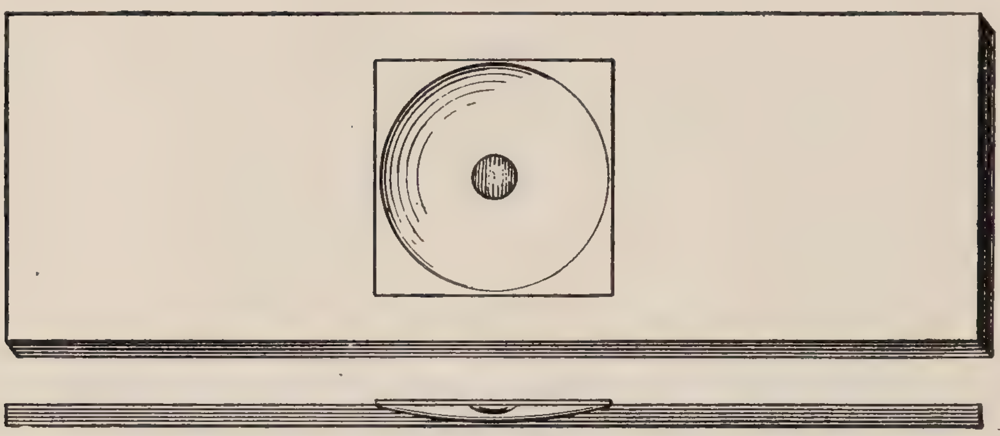


FIG. 22.—The "hanging drop" seen from above and in profile.

then examined by focusing upon it with the oil-immersion lens, a small aperture of the iris diaphragm of the condenser being used to render the bacteria visible by refraction. To facilitate focusing, the edge of the drop should be brought into the center of the field of the low-power objective, and then the oil-immersion put in place and focused upon it, the edge of the drop being more readily seen as a sharp line, owing to refraction, than the organisms. Great care is necessary to avoid breaking the cover-glass in the effort to bring the bacteria into view. Hanging drops may also be prepared from suspensions of bacteria grown on solid media, by mixing a portion of the growth with a small quantity of bouillon.

In the study of spore-formation the hanging drop is of great utility. Here the slide and cover-glass must be carefully sterilized before using, the cavity between the cover-glass and the slide well sealed with vaselin, and other precautions taken to prevent contamination of the drop with other bacteria. The preparations may be placed in the incubator or on a "warm stage" and the process of spore-formation followed.

H. W. Hill's "Hanging-block" Method for the Observation of Developing Bacteria.—"Pour melted nutrient agar into a Petri dish to the depth of about one-eighth to one-quarter

inch. Cool this agar and cut from it a block about one-quarter inch to one-third inch square, and of the thickness of the agar layer in the dish. This block has a smooth upper and under surface. Place it, under surface down, on a slide, and protect it from dust. Prepare an emulsion in sterile water of the organism to be examined if it has been grown on a solid medium or use a broth culture; spread the emulsion or broth upon the upper surface of the block as if making an ordinary cover-slip preparation. Place the slide and block in a 37° C. incubator for five or ten minutes to dry slightly. Then lay a clean sterile cover-slip on the inoculated surface of the block in close contact with it, usually avoiding air-bubbles. Remove the slide from the lower surface of the block, and invert the cover-slip so that the agar block is uppermost. With a platinum loop run a drop or two of melted agar along each side of the agar block, to fill the angles between the sides of the block and the cover-slip. This seal hardens at once, preventing slipping of the block. Place the preparation in the incubator again for five or ten minutes to dry the agar seal. Invert this preparation over a moist chamber and seal the cover-slip in place with white wax or paraffin. Vaselin softens too readily at 37° C., allowing shifting of the cover-slip. The preparation may then be examined at leisure. For bacillus diphtheriæ and organisms of similar size, Zeiss ocular 5, objective $\frac{1}{12}$, oil immersion, and a Welsbach light prove satisfactory, although a lower ocular and higher objective are better. The Abbé condenser is not used. If preferred, the Welsbach light may be concentrated by a four-inch lens, focal length seven inches. An incandescent electric lamp is very difficult to focus and does not yield good results." . . .

"Bacteria multiplying readily at room-temperature can be observed in such a preparation exactly as an ordinary hanging drop is observed, except that the slide should be secured rigidly in some way to the microscopic stage to prevent shifting. For bacteria growing best at 37° C. a warm stage is required." [1]

Celloidin Sacs.—By reason of the osmotic property of the celloidin these sacs permit the study both of the action of the body-fluids of an animal on bacteria contained in them and of the action of the metabolic products of the bacteria on the animal, without permitting the bacteria to invade the tissues.

The method of Harris [2] for preparing these sacs seems to be an improvement on the method of McCrae, and is as follows:

Affix a piece of glass tubing, 4 cm. long and 3 mm. in

[1] *Journal of Medical Research*, vol. vii., pp. 202–212. In this paper there are also described forms of warm stages and the method of using them.

[2] *Centralbl. f. Bakt.*, 1. Abth., Orig. 1902, vol. xxxii., p. 74.

inside diameter, by heating to the smaller end of a large gelatin capsule (Parke, Davis & Co. "No. 12 Veterinary"). Remove any gelatin from the lumen of the tube, and paint the joint with a moderately thick celloidin. When dry, dip the capsule in thin celloidin beyond the junction with the glass tube, and rotate in the air until dry, to obtain an even coating. Do this repeatedly until a coating of the desired thickness is obtained. When thoroughly dry, apply thicker celloidin to the joint, to the other pole of the capsule, and to the line of junction of the two portions of the capsule, and allow to dry thoroughly. The gelatin is then to be removed by filling the capsule with water with the aid of a small Pasteur pipet and placing the capsule in boiling water for a few minutes, after which the melted gelatin and water is removed with the pipet. The sac is then filled with bouillon and placed with the glass tube downward in a test-tube containing sufficient bouillon to cover the sac to the depth of 1 cm., and the whole sterilized five minutes in the autoclave or in the Arnold steam sterilizer in the usual manner. Before placing the bacteria in the sac, some of the bouillon is removed under aseptic precautions by means of a pipet, so that a small empty space in the sac results. The bacteria are introduced with a pipet and the glass tube, grasped by sterile forceps near the junction with the sac, is sealed off in the small flame of the blast-lamp. The sac is then washed in sterile water and placed in a bouillon culture-tube and incubated overnight to test its permeability for bacteria. If the bouillon outside of the sac remains sterile, the sac is then ready to be placed in the peritoneal cavity of the animal.

3. THE INOCULATION OF ANIMALS.

The animals ordinarily used in the laboratory are guinea-pigs, rabbits, and mice. The instruments, etc. used in the inoculation of animals should be sterilized beforehand, but strict surgical asepsis is not necessary as a rule.

Guinea-pigs are in most instances inoculated either subcutaneously or into the peritoneum.

Subcutaneous inoculation is effected either by injection with

a hypodermic syringe or by the introduction of the material to be inoculated through a small incision in the skin. The best point for subcutaneous inoculation is the tissue of the anterior abdominal wall.

In inoculating, the animal is to be held abdomen uppermost by an assistant, who grasps the neck and fore quarters with one hand and the hind quarters with the other. If the skin is to be incised, the hair about the point of inoculation is to be cut short with a pair of scissors and the skin cleansed with soap and water. An incision is then to be made about 8 or 10 mm. long through the skin, including the subcutaneous tissue, and the superficial tissues separated from the muscle for a distance of 10 or 15 mm. toward one side of the wound by inserting the points of scissors or other instrument, so as to form a "pocket" beneath the skin. In this "pocket" the material for inoculation is introduced, either on the platinum wire (see page 98) or by means of small forceps.

If pieces of tissue are used, it may be well in some cases to close the wound by one or two sutures in order to prevent the extrusion of the material after the release of the animal.

Intraperitoneal inoculation may be performed essentially as above indicated. If the inoculation be by incision, the opening into the peritoneal cavity should be as small as possible, and the wound should be firmly closed with silk sutures in order to prevent extrusion of the intestines.

In inoculating with the hypodermic syringe the needle should not be pushed in too far or the intestines may be wounded. The needle is best introduced a little to one side of, or slightly below, the umbilicus.

Rabbits.—These animals may be inoculated both subcutaneously and intraperitoneally, essentially as described for guinea-pigs.

In lifting or in carrying rabbits from one place to another the animals are to be grasped by the ears. During the operation of inoculating, the assistant grasps the ears with one hand and the hind legs with the other, while the body of the animal rests upon the table, abdomen uppermost. Rabbits

held for a few seconds in this position usually become perfectly quiet, and often do not show any evidence of pain during the operation.

Intravenous inoculation is usually done on rabbits, because of the ease with which the needle of a hypodermic syringe may be introduced into the long and prominent marginal vein of the ear. In inoculating in this manner the tip of the ear is held by the thumb and fingers of the left hand, while the right manipulates the syringe, the needle of which is pushed through the skin of the external surface of the ear into the vein which runs along the outer margin of the ear (Fig. 23).

By the exercise of care and gentleness the animal may be thus inoculated without being held by an assistant, especially if the fur between the ears be stroked for a short time just before the introduction of the needle. In some cases it may be necessary to anesthetize the animal on account of violent struggling. (See below.)

Injection of bacteria into the mesenteric veins by means of the hypodermic syringe, after laparotomy, may be performed both on rabbits and on guinea-pigs. This is to be done under anesthesia. Ether is very satisfactory for this purpose. Guinea-pigs bear it well, but it is to be used with caution on rabbits. With the latter animals death is liable to occur if the ether is "pushed" after complete anesthesia is established. Rabbits once thoroughly anesthetized seem to remain so for a considerable time without additional ether being necessary. The incision for this form of inoculation should be in the lower half of the abdominal wall in the median line, for in this region the coils of the small intestine are most numerous. The length of the incision should be about 2 cm. Several loops of intestine are brought out through the wound, and a mesenteric vein, of the proper size to admit the needle of a hypodermic syringe, is sought for. When found the needle is to be introduced and held firmly in position while an assistant carefully presses inward the piston of the syringe. After the injection of the material the needle is withdrawn, the punctured vein picked up with the artery-forceps, and the vessel tied on both sides of the puncture with silk thread. The loops of the intestine are then replaced and the wound closed in two layers, one consisting of the muscles and peritoneum, the other of the skin. The so-called "button-hole stitch" with silk thread is very well fitted for the closing of the wound.

Little or no aseptic precautions are necessary to obtain primary union in the wound. Before the operation, however, the hair of

the region should be cut off close and the skin cleansed with soap and water.

This form of inoculation may be useful in studying the local effects of bacteria upon liver-tissue, for large numbers of them

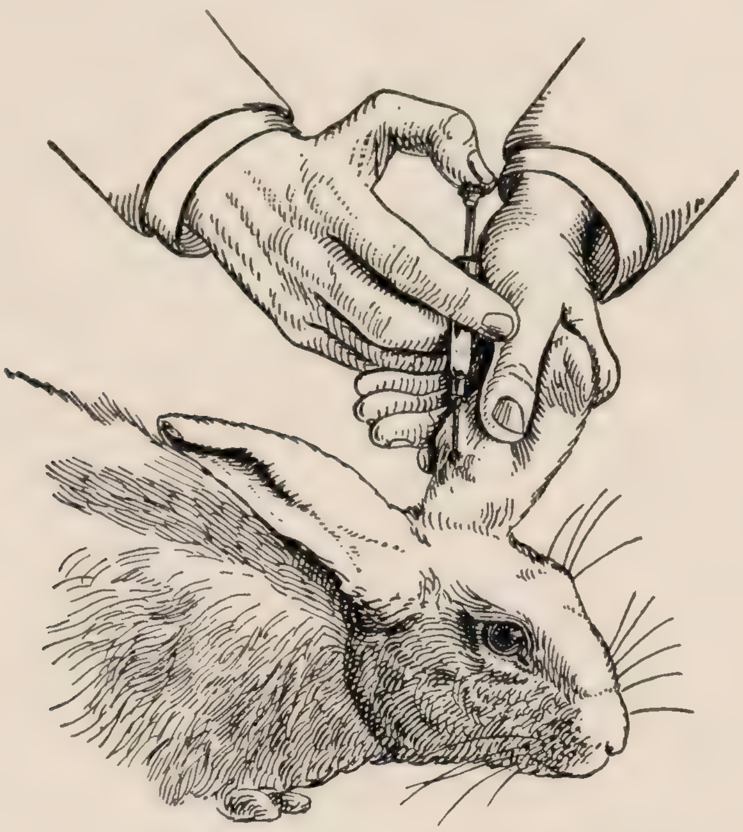


FIG. 23.—Method of making an intravenous injection into a rabbit. Observe that the needle enters the posterior vein from the hairy surface (McFarland).

will be lodged in the capillaries of the liver, and microscopical sections of any part of the organ will contain them, so that any local lesion produced by them may be subjected to observation after variable intervals of time.

Mice are usually inoculated subcutaneously at the root of the tail. The animal, manipulated by means of chemists' crucible tongs or a similar instrument grasping his tail, is to be persuaded to crawl into a cylinder of wire gauze, about 8 to 10 cm. long and about 3 cm. in diameter, which is fixed on a small board. The cylinder is open at both ends, and when the mouse has crawled into it—a thing which he will readily do—the end near his tail is bent inward so as to prevent him from backing out of it, while an ordinary small screw-clamp is adjusted firmly to his tail to prevent his escaping through the other end. The animal is thus secured and ready for the operation of inoculation. A more complete form of this apparatus, with a fixed clamp for the animal's tail, is shown in Fig. 24.

In making the inoculation the mouse is pulled backward

by the tail until his rump is exposed in the end of the cylinder, and then with small scissors the hair is cut away over a space, approximately 1 cm. square, about the root of the tail. In the center of this a small opening is made through the skin 3 or 4 mm. long with small scissors, and through the opening the points of the scissors are passed anteriorly beneath the skin for a distance of about 1 cm., so as to make a "pocket" or cavity by separating the skin from the muscles. Into the cavity thus formed the material for inoculation is then to be introduced by means of the platinum wire. As a rule, white mice are to be preferred to the wild brown variety, on account of the greater ease with which they may be handled.

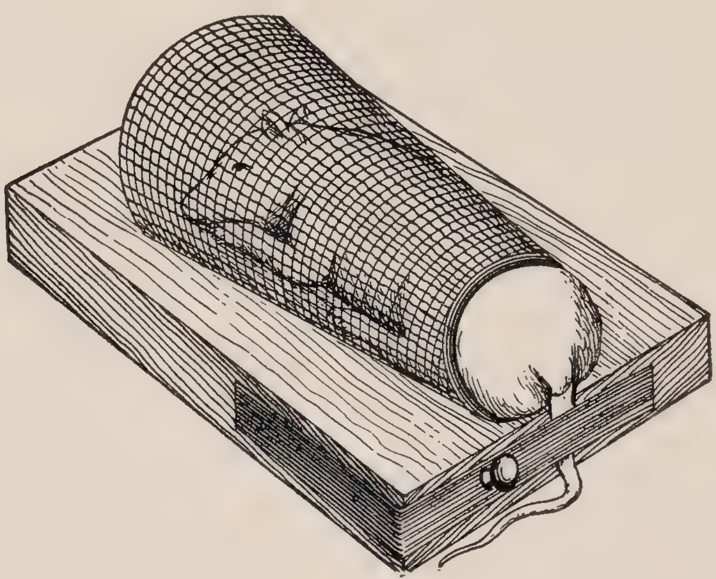


FIG. 24.—Mouse-holder, with mouse in position for inoculation.

Mice may also be inoculated in the peritoneal cavity by introducing a very few drops of a suspension or a bouillon culture of an organism with a hypodermic syringe.

The quantity of bacteria used for purposes of inoculation varies with the organism and with the end in view. In general, it may be said that in inoculating with the growth from a solid medium with the platinum wire one or two loopsful are used. If bouillon cultures are employed, the quantity injected varies from $\frac{1}{10}$ c.c. to 1 c.c. in most cases.

In cases where a "suspension" of the growth on a solid medium is injected the same quantities are used as in the case of bouillon cultures, the density of the suspension depending upon the operator. A "suspension" may be con-

veniently prepared by pouring 5 or 8 c.c. of sterile bouillon, sterilized water, or 0.6 per cent. sodium chlorid solution (sterilized) into the tube containing the growth upon solid medium, then breaking up the colonies of the growth with the platinum wire, and shaking the tube.

The Care of Animals.—Inoculated guinea-pigs should be kept in boxes or cages so arranged as to permit of cleaning and disinfection. Cages made of a combination of galvanized-iron wire netting and galvanized sheet iron are to be preferred. The bottom of the cage should contain sawdust, and the top may be made to open ou hinges. Good dimensions for such cages are 16 inches long, 10 inches wide, and 10 inches high. They may be satisfactorily disinfected, in most instances at least, by washing with boiling water.

Inoculated mice are well kept in large glass jars with perforated covers. A small amount of raw cotton should be provided for bedding.

The "stock" guinea-pigs and rabbits may be kept together in a pen which should have light and ventilation. Guinea-pigs breed readily and their young thrive, but this is not usually the case with rabbits. Mice may be kept for use in a woven-wire cage set in a sheet-iron pan, which will permit of the easy removal of excreta. Some raw cotton should be furnished for bedding. The young of white mice are difficult to raise to maturity.

Food.—Rabbits and guinea-pigs eat the same things. In summer-time, grass, green corn-husks, and green vegetables generally are good food for them. In winter, carrots and oats form a satisfactory diet. Fresh water should also be supplied.

Mice may be fed on stale bread soaked in water, oats, bird-seed, and occasionally some cheese. Fresh water should be furnished, and, if possible, a little milk sometimes.

4. CULTIVATION WITHOUT OXYGEN (ANAËROBIC CULTURES).

Of the numerous methods and modifications of methods that have been proposed for the cultivation of anaërobic bacteria, only those are given here which have worked suc-

cessfully in our hands, or are regarded as the simplest and most practical.

Culture-media for Anaërobic Bacteria.—The agar-agar, gelatin, or bouillon used for the cultivation of anaërobic bacteria should contain 1 per cent. glucose. These media should not be more than two weeks old for the best results.

Their reaction is of the greatest importance, and should be adjusted by titration (see page 84). In the case of gelatin and of agar-agar the reaction should be 1 per cent. or 1.5 per cent. of normal acidity to phenolphthalein. In the case of glucose bouillon, however, a more rapid growth is obtained with a reaction of less than 1 per cent. normal acidity to phenolphthalein. A degree of acidity greater than this is probably a frequent cause of failure to obtain cultures of obligate anaërobes in bouillon. Therefore, it is of the utmost importance, in working with glucose bouillon, to be sure that it has the proper reaction at the time of its use.

The culture-medium must be thoroughly boiled immediately before inoculation in order to expel absorbed oxygen. It is then to be cooled rapidly by immersing the tube in cold water, and is to be inoculated within a few minutes afterward.

Method of Liborius (Fig. 25).—This consists in cultivating the bacteria in the depths of solid media in test-tubes filled to a considerable height, so that oxygen cannot penetrate to them through the thick layer of medium.

A test-tube is filled about three-quarters full[1] of sterile glucose gelatin or glucose agar-agar, and its contents boiled for a few minutes to expel the excess of oxygen from the medium. The tube is then immersed in cold water to cool its contents rapidly, and then, before the medium becomes solid, the tube is placed in a water-bath at 38° to 40° C. for a few minutes. When the medium may be assumed to have reached this temperature, it is inoculated with the material from which a growth is sought to be obtained, and then rapidly solidified in cold water. The colonies of anaërobic

[1] The tube need not be filled more than half its length.

bacteria develop only in the deeper layers of the culture-medium. These colonies may bė made accessible for sub-cultures either by breaking the tube or by removing the overlying portions of the culture-media by means of a stout platinum wire, previously sterilized in a flame. For taking out colonies for transplantation, a capillary glass tube, sterilized in a flame, may be found useful in place of the platinum wire. In inoculating the tube, care should be taken to secure a good distribution of the bacteria through the medium by manipulating the platinum wire.

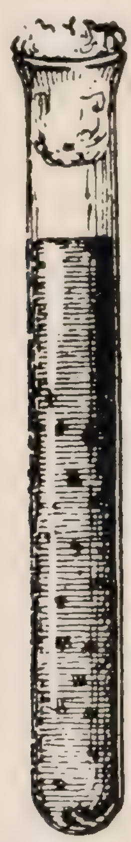


FIG. 25.—Liborius's method of making anaërobic cultures.

FIG. 26.—Buchner's method of making anaërobic cultures.

This method will be found very practical for obtaining pure cultures from mixed growths if dilutions (see page 112) be made. In making dilutions it is well to use a tube of bouillon or sterilized water for the first tube, thus economizing medium, for the first tube will usually have so many colonies that no colonies suitable for sub-cultures will be available.

The microscopical appearances of the colonies may be studied by placing thin slices of the medium, containing the colonies, on a slide. These slices may be easily ob-

tained with the aid of a stout platinum wire with a flattened end, more or less bent.

Anaërobic bacteria grow readily in "*deep stab*" *cultures.* In these cultures the medium should fill the tube to almost half its height at least. After inoculation some melted medium may be poured in so as to fill the tube to an additional height of some centimeters, but this is not necessary.

Simple Anaërobic Plate-cultures.—These are prepared like the ordinary Petri plate-cultures (see page 113) except that the melted culture-medium is poured into the upturned larger dish, or cover, of the pair, while the smaller dish is then placed, bottom surface downward, in the melted culture-medium, and allowed to settle by its own weight into the fluid medium. The dishes are not disturbed until the medium has hardened. Sufficient medium should be used to fill the space between the sides of the dishes. This quantity will be about 10 c.c. By slightly inclining the smaller dish in placing it in the melted medium, air-spaces can be easily avoided.

By this method the colonies develop in a thin layer of culture-medium enclosed between glass surfaces. The method gives a good chance to study the microscopical characters of the colonies. Surface colonies are, of course, not obtained by this method. The colonies are easily made accessible for transplantation by separating the dishes from one another. The layer of culture-medium will adhere to one dish or the other.

In order to avoid contamination, the dishes should be arranged in the manner above described during their sterilization previous to using.

Buchner's Method.—This method consists in cultivating bacteria in an atmosphere from which the oxygen has been absorbed by a mixture of alkali and pyrogallic acid. Tube-cultures, or cultures in Petri dishes, may be used. They should be placed in some form of a glass chamber, which is closed air-tight, along with the necessary quantity of alkali and pyrogallic acid mixture. In preparing the apparatus, the pyrogallic acid (in powder) is placed first in the chamber along with the culture tubes or plates, then the necessary

quantity of a solution of potassium hydroxid (1 : 10) is run in, and the chamber quickly closed. For single tube-cultures a large test-tube provided with a tightly fitting rubber stopper, which is sealed in position with wax, may be used for the air-tight chamber (see Fig. 26). The culture-tube is to be elevated above the surface of the reducing mixture by means of a bent wire.

If a number of tube-cultures or Petri plate-cultures are desired, the glass chambers known as Novy's jars are very satisfactory to use. The joints of this apparatus should be well smeared with vaselin. To avoid breakage the test-tube containing the inoculated culture-medium may be held in a beaker, with some cotton at the bottom, while in the apparatus. Petri plate-cultures may be placed one above another in the jar, the bottom plate being supported above the level of the reducing fluid by some sort of wire frame.

It is necessary to seal up the apparatus quickly in order to obtain the full benefit of the oxygen-absorbing power of the pyrogallic acid. The quantity of pyrogallic acid employed should be about 1 gram for each 100 c.c. of air-space to be exhausted of oxygen, and for every gram of pyrogallic acid 10 c.c. of the solution of potassium hydroxid should be used.

Hans Zinsser's Method for Anaerobic Plate-cultures.— The method is described by Dr. Zinsser as follows:

"The apparatus used consists of two circular glass dishes, fitting one into the other, as do the halves of a Petri dish, and similar to these in every respect except that they are higher, and that a slightly greater space is left between their sides when they are placed together. The dishes should be about $\frac{3}{4}$ to 1 inch in height; they need be of no particular diameter, although those of about the same size as the usual Petri dishes are most convenient. The sole requirement necessary for successful plating is that the trough left between the two plates when put together shall not be too broad, a quarter of an inch being most favorable.

"Into the smaller of these plates the inoculated agar is poured, exactly as is done into a Petri dish in the ordinary

aërobic work. Prolonged boiling of the agar before plating is not essential. When the agar-film has become sufficiently hard on the bottom of the smaller dish, the entire apparatus is inverted. The smaller dish is now lifted out of the larger, and placed, still inverted, over a moist surface—a towel or the wet surface of the table—to prevent contamination. Into the bottom of the larger dish, which now stands open, there is placed a quantity (1 to 2 drachms) of dry pyrogallic acid. Into this, over the pyrogallic acid, the smaller dish, still inverted, is then placed. A strong solution of sodium hydrate is poured into the space left between the sides of the two dishes, in quantity sufficient to fill the receiving one half full. While this is gradually dissolving the pyrogallic acid (and this is the only step which requires speed), albolene, or any other oil, is dropped from a pipette, previously filled and placed in readiness, into the same space, thus completely sealing the chamber formed by the two dishes.

"If these steps have been performed successfully, the pyrogallic solution will at this time appear of a light brown color, and the smaller plate, with its agar-film, will float unsteadily above the other. Very rapidly, as the pyrogallic acid absorbs the free oxygen in the chamber, this plate is drawn down close to the other, and the acid assumes a darker hue, which remains without further deepening even after three or four days' incubation."

Wright's Method.—The method depends upon the absorption of oxygen by an alkaline solution of pyrogallic acid, as in the well-known method of Buchner. It is applicable to culture in test-tubes and in flasks. The details of the method are as follows:

After the culture-medium in the test-tube has been inoculated, the cotton stopper is thrust sufficiently far down into the test-tube so that the upper end of the cotton stopper lies about 15 mm. below the mouth of the test-tube. It is usually desirable to cut off a part of the protruding portion of the cotton before doing this. Now fill the space in the tube above the cotton stopper with dry pyrogallic acid. Next pour quickly onto this pyrogallic acid enough of a strong watery solution of sodium hydrate to dissolve it all;

avoid pouring on an excess; for a test-tube ¾ of an inch in diameter about 2 c.c. will be an ample quantity. Then, as quickly as possible, insert firmly a rubber stopper in the mouth of the tube so as to close it tightly. The culture is then ready to be set aside for development.

The cotton of the stopper should be of a kind that will readily absorb fluids.

The solution of sodium hydrate consists of one part of sodium hydrate in sticks and two parts of water.

It may be thought that there is danger of contaminating the culture-medium from the alkaline pyrogallic acid mixture running down the sides of the tube. This does not occur, because the mass of the cotton stopper is sufficiently large to absorb completely the quantity of fluid in it, with a good margin to spare.

This simple method has given satisfactory cultures of the tetanus bacillus obtained from cases of tetanus in the Massachusetts General Hospital and of other obligate anaërobic bacteria. It can be applied to all forms of test-tube cultures, both in solid and fluid media, including Esmarch roll-cultures. In applying the method to Esmarch roll-cultures the mixture of pyrogallic acid and alkali should be placed in the cotton, and the rubber stopper inserted before the tube is rolled on the ice. Glucose-agar readily lends itself to Esmarch roll-cultures if the tubes are kept in a slanting position during growth.

FIG. 27.—Wright's method for the cultivation of anaërobes.

The accompanying photograph shows the appearance of a bouillon tube prepared according to this method (Fig. 27).

IV. SPECIAL BACTERIOLOGY.

The number of species of bacteria of pathogenic significance which are *commonly* encountered in pathological processes in man is a small one. These comprise the staphylococcus pyogenes aureus, the streptococcus pyogenes, the pneumococcus, the bacillus coli communis, the typhoid bacillus, the bacillus diphtheriæ, and the bacillus tuberculosis. It is with infections with these few species that the pathologist is most frequently concerned, and the determination of the presence of these alone comprises by far the greater part of the bacteriological work which he is called upon to do.

In the following descriptions of the important pathogenic bacteria which are concerned in human pathology the main object will be to give those characteristics which will serve for their identification, rather than an exhaustive consideration of their various properties and modes of growth.

Staphylococcus Pyogenes Aureus.—The colonies on blood-serum are golden yellow in color. They are rounded, shining, slightly elevated, and may attain a diameter of 2 mm. or more after remaining for thirty-six hours in the incubator. The color of the colonies varies from a pale yellow to a deep orange. Young colonies may be creamy white, becoming yellow later.

Morphology.—Rather small cocci, frequently arranged in masses or clumps.

Stained by Gram's method.

Gelatin Stab-culture.—Growth along the line of stab, followed by liquefaction in funnel form, with yellow sediment and clouding of the liquefied medium (Fig. 29).

Potato.—Yellow confluent colonies.

Agar-agar Slant.—Rather broad shining streak with sharply defined margins, at first white in color, but later becoming yellow.

Bouillon.—Densely clouded. A yellowish sediment is formed, and sometimes a thin pellicle is seen on the surface

Litmus-milk.—Turned pink and coagulated.

Pathogenesis.—When inoculated into the circulation of a rabbit death follows in from eighteen hours to three days in the case of virulent cultures. Not all specimens of this organism are virulent. The lesions produced in the rabbit by inoculation in the ear-vein in typical cases are abscesses with infarctions in the kidneys, and miliary abscesses in the

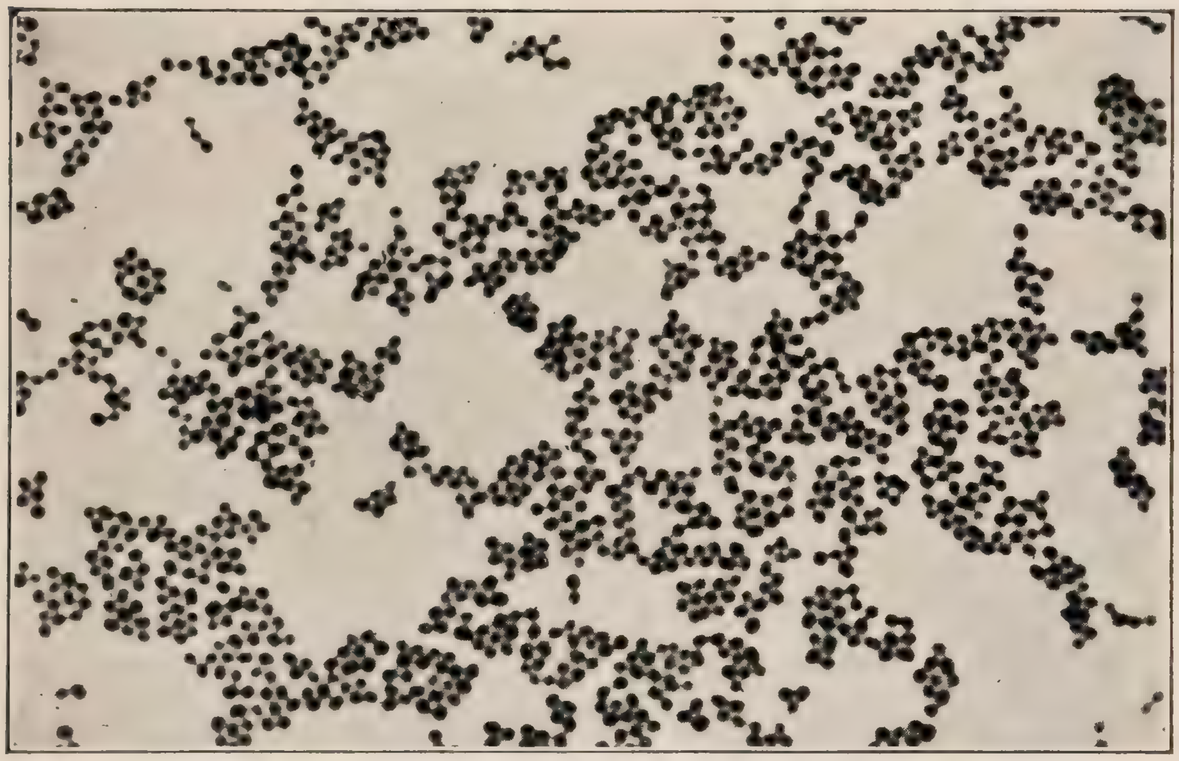


FIG. 28.—Staphylococcus pyogenes aureus from a culture; × 2000 (Wright and Brown).

myocardium, diaphragm, and voluntary muscles. In the kidneys lines of necrosis with purulent infiltration, mainly in the pyramids, are frequently observed. This organ is the one most constantly affected. The number and extent of the lesions vary in different animals and with different cultures. They are best developed in animals which survive about three days. In animals which succumb after eighteen hours no macroscopic change may be apparent. On microscopical examination of the kidneys, however, small areas of necrosis will usually be found, mainly in the pyramids, surrounding masses of cocci. In the kidneys of animals which survive longer all the grades of invasion of these necrotic areas by leucocytes, up to regular abscess-formation, may be traced. By cultures the organism will be found in large

numbers in the kidneys and urine of the rapidly fatal cases, and in smaller numbers in the other organs and blood of the heart.

Occurrence.—The staphylococcus pyogenes aureus is found most commonly in pus-formations of a circumscribed character and also in a large number of pathological conditions, of which only the more important will be mentioned here.

These are as follows: Osteomyelitis, peritonitis, pleuritis,

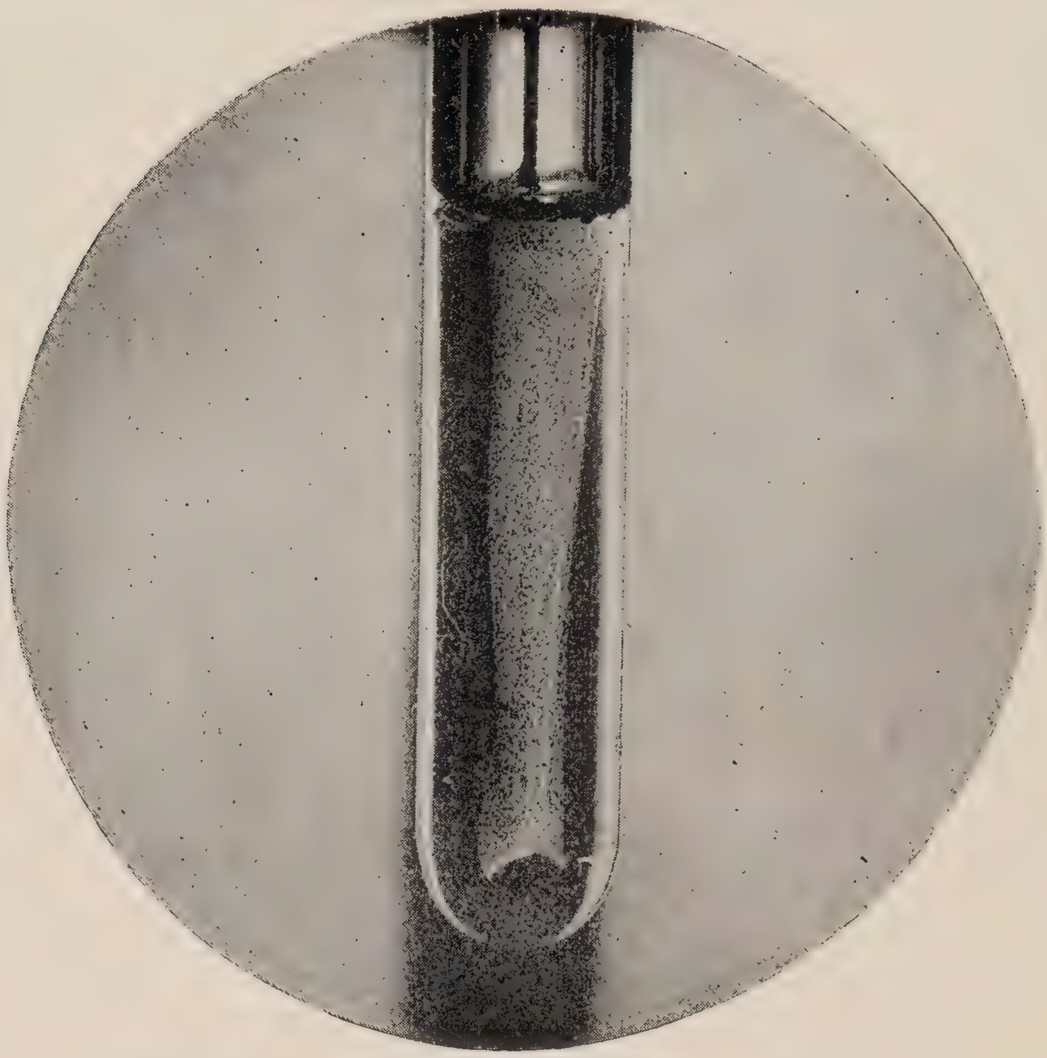


FIG. 29.—Staphylococcus pyogenes aureus: stab-culture three days old in gelatin (Fränkel and Pfeiffer).

endocarditis, meningitis, broncho-pneumonia, and puerperal septicemia. It may also be found in the blood of the various internal organs at autopsies in cases in which a suppurative or other acute inflammatory process is present anywhere, with or without metastatic abscess-formation. The organism also occurs frequently in the dust of places inhabited by man, as well as on the surface of the skin and of the mucous membranes of the nose and mouth.

Diagnosis.—The staphylococcus pyogenes aureus cannot usually be identified with any certainty by the cover-glass examination alone. Cultures are necessary in order to differentiate from the other staphylococci and from the streptococcus.

For practical purposes the identification of the pyogenic cocci may be made by the appearances of their colonies on blood-serum and by their morphology; no secondary cultures are usually necessary.

The following staphylococci may also be present in acute inflammatory processes, but they occur less frequently than does the staphylococcus pyogenes aureus.

Staphylococcus Pyogenes Albus and Staphylococcus Pyogenes Citreus.—These organisms differ from the staphylococcus pyogenes aureus mainly in the color of their colonies. As a rule, they are much less pathogenic for rabbits than that organism.

Staphylococcus Epidermidis Albus (Welch).—"Is probably only a variety of the staphylococcus pyogenes albus. Usu-

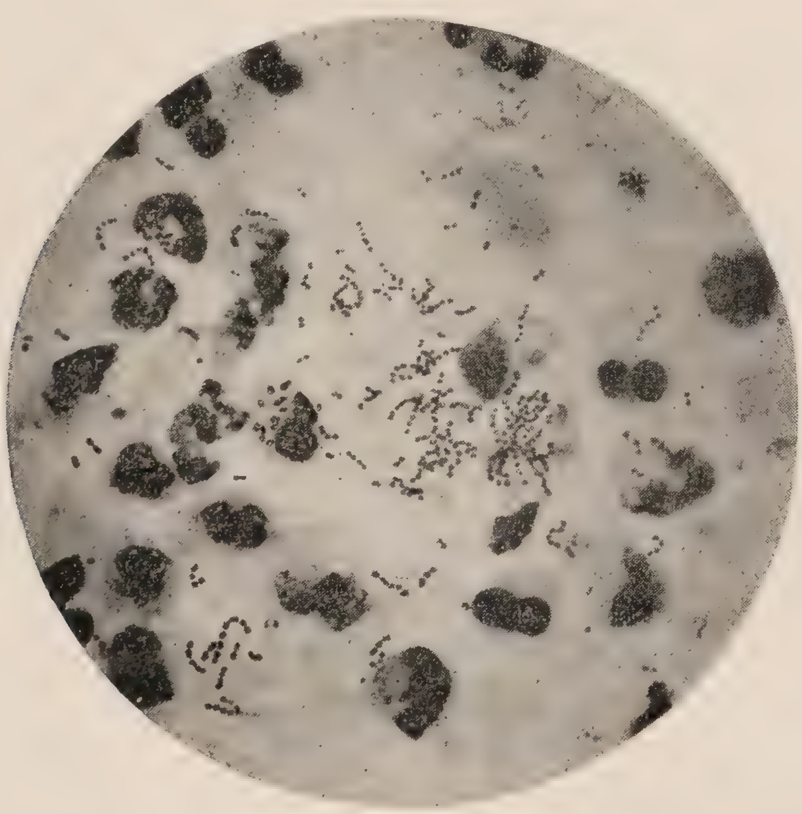


FIG. 30.—Streptococcus pyogenes; cover-glass preparation of the pus of an abscess; × 1000 (Fränkel and Pfeiffer).

ally grows somewhat more slowly; liquefies gelatin and coagulates milk less rapidly. Is of little virulence under ordinary conditions. Is a regular inhabitant of the epidermis, lying deeper than can be reached by disinfection of the surface of the skin" (Welch).

Staphylococcus Cereus Albus.—Very similar to the staphylococcus pyogenes albus, but does not liquefy gelatin. May occur in abscesses.

Staphylococcus Cereus Flavus.—This organism is similar to the preceding, except that it forms a lemon-yellow pigment.

Streptococcus Pyogenes and Allied Streptococci.—Different strains of streptococci show considerable variation in their biological properties. It is not certain whether those concerned in pathological processes are of various species or only modifications by environment of one species.

Blood-serum.—Minute grayish-white colonies, often looking like small grains of sand scattered over the surface of the medium. Sometimes the colonies are shining, translucent, colorless, resembling minute dewdrops.

Morphology.—Rather small cocci arranged in chains, many of the cocci being divided into two hemispheres by a line of division running at right angles to the axis of the chain. In some strains the cocci are conical or oval in outline. The chains may be made up of many cocci and be quite long.

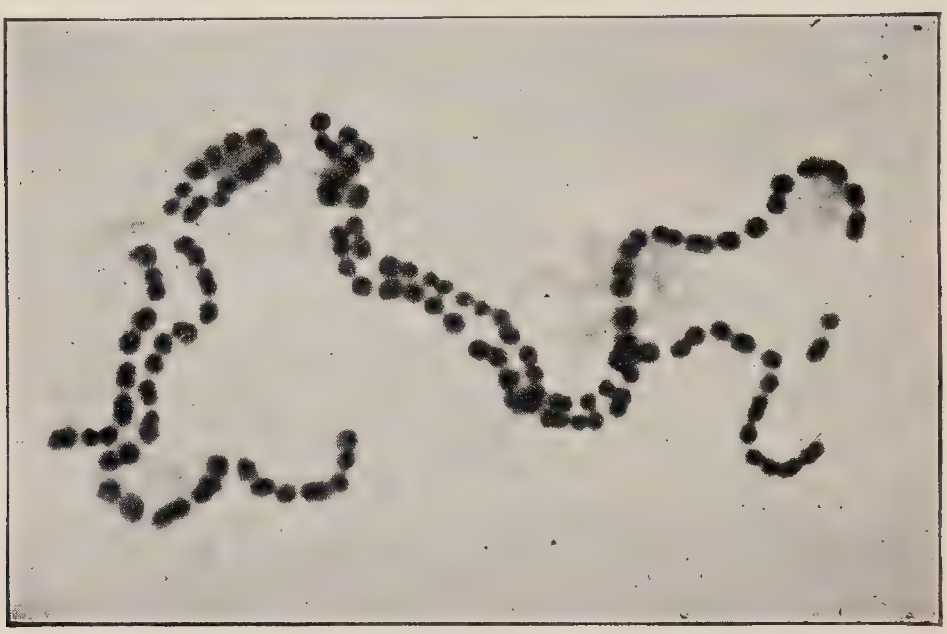


FIG. 31.—Streptococcus pyogenes from a culture in bouillon; × 2000 (Wright and Brown).

Cover-glass preparations from the colonies often fail to show the characteristic chain arrangement, owing to the chains being broken up by the manipulation. The chain-formation is best demonstrated in cover-glass preparations from the "water of condensation" at the bottom of the blood-serum tube. This is essentially a bouillon culture,

and it is in such fluid media that the chain-formation is best developed. In preparing the cover-glass from this as little manipulation of the fluid as possible should be used, in order to avoid destroying the chain arrangement.

Practically, the only organism with which the streptococcus may be confounded is the pneumococcus, which also grows in minute colonies and sometimes in chains. The streptococcus may be distinguished from the pneumococcus, however, by the morphology of the individual organisms, the streptococci appearing as pairs of hemispheres, and the pneumococci as pairs of oval, conical, or lancet-shaped organisms, the broader ends of which are in apposition.

Stains by Gram's method.

Bouillon.—The character of the growth in bouillon is subject to considerable variation, and certain doubtful *varieties* of the streptococcus are distinguished mainly by the bouillon culture.

"We thus distinguish short-chained streptococci ('streptococcus brevis'), long-chained streptococci ('streptococcus longus'), streptococci which render bouillon cloudy and those which do not, streptococci which form flocculent or scaly or sandy or viscous sediments.

"The name 'streptococcus conglomeratus' is given to a streptococcus which grows, without clouding the bouillon, in the form of dense, separate particles, scales, or thin membranes at the bottom or sides of the tube, and on shaking the sediment it breaks up into little specks, without producing uniform diffuse cloudiness.

"On microscopical examination the chains in the latter case are long and interwoven in conglomerate masses. Streptococcus chains may be straight or wavy or twisted. These various distinctions are only of relative value. One form may change into another. Virulent streptococci may be found among all the groups mentioned; the streptococci of erysipelas and most of the streptococci from abscesses and septicemia grow in long chains in bouillon" (Welch).

Ascites Dextrose Bouillon.—This consists of 1 part of sterile ascitic fluid mixed under aseptic precautions with 3

or 4 parts of sterile 1 per cent. dextrose bouillon. In this medium the streptococci grow more abundantly than in ordinary bouillon.

Agar-agar Slant.—Minute grayish translucent colonies (Fig. 32).

Agar-agar Stab.—Small spherical grayish colonies along the needle-track.

Gelatin.—Growth similar to that on agar-agar.

Litmus-milk.—Some varieties turn the medium pink and cause coagulation.

FIG. 32.—Streptococcus pyogenes: culture upon agar-agar two days old (Fränkel and Pfeiffer).

Fermentation of Carbohydrates.—Great differences exist among various strains in the ability to ferment certain carbohydrates, such as lactose, mannite, inulin, salicin, dextrose, and saccharose. One or more of these or other carbohydrates may or may not be fermented by a given strain.

For testing fermentation, *Hiss's serum-water medium* may be used. This consists of a mixture of 1 part of beef serum and 2 or 3 parts of distilled water, to which 1 per cent. of a 5 per cent. solution of purified litmus is added. This mixture is heated to 100° C. for a few minutes, and then the carbohydrate desired is added to it in the proportion of 1 per cent., after which it is distributed in fermentation tubes and sterilized in the usual manner. Instead of the fermentation tubes, Durham's tubes may be used. These are ordinary culture-tubes, in each of which is placed, in inverted position, a small test-tube about 7 cm. long and 8 mm. in diameter. The outer tubes are filled with the medium to a height of about 5 cm. During sterilization the air in the inner tubes is driven out, and, upon cooling, the medium takes its place.

Fermentation is indicated by the appearance of gas in the inner tube.

Hemolysis.—Some strains hemolyze human red blood-corpuscles and some do not. Hemolyzing power is tested by growing colonies upon the surface of blood-agar plates. Hemolysis is shown by the appearance of a clear zone about the colony. Some strains cause a greenish coloration of the surrounding medium, and are called by some writers *Streptococcus viridans.* The blood-agar plates may be made as described for hydrocele fluid-agar plates on page 147. Sterile defibrinated human blood is used instead of hydrocele fluid.

Pathogenesis.—The results of the inoculation of animals are not constant, great variation in the virulence of different cultures being observed. Sometimes mice inoculated at the root of the tail or in the peritoneal cavity will die in about twenty-four hours with enlargement of the spleen and large numbers of the organism in the internal organs.

Some strains produce arthritis in rabbits by intravenous inoculation. E. C. Rosenow has shown also that the *Streptococcus viridans* may produce vegetative endocarditis in these animals. He injected intravenously large quantities of the micro-organism obtained by centrifugalization of cultures in ascites-dextrose bouillon, and used young animals. With strains from the joints of acute articular rheumatism he produced arthritis, endocarditis, pericarditis, myocarditis, myositis, and other lesions.

Occurrence.—The streptococcus occurs frequently in the spreading phlegmonous inflammations as well as in suppurative processes generally, and is the most common cause of septicemia. It is almost always present in inflammatory conditions of the mucous membrane of the pharynx, and is often encountered in bronchopneumonia. In erysipelas it is almost invariably the infecting organism, and it is the most frequent cause of puerperal septicemia. In the joints of acute rheumatism the streptococcus has been demonstrated. In the majority of fatal cases of diphtheria and in some cases of typhoid fever, scarlet fever, tuberculosis, and other acute inflammatory diseases it will be found in

the blood of the various internal organs after death. It also occurs in a certain proportion of cases of peritonitis, pleuritis, meningitis, endocarditis, and otitis media. In endocarditis, masses of the micro-organisms may form a large part of the substance of the cardiac vegetations. Gaining entrance to the tissues through an insignificant wound or abrasion of the skin, it may produce a rapidly fatal septicemia in a susceptible individual, in whose internal organs at autopsy large numbers of the organism will be found. This general invasion of the circulation may also be observed in cases of chronic or wasting disease, the infection occurring during the last days or hours of life (terminal infection).

Of other conditions in which it may occur, hepatic abscess, appendicitis, osteomyelitis, and synovitis may be mentioned. Although the streptococcus is distinctly one of the pus-producing bacteria, yet the inflammations of the soft parts of the extremities which are produced by it are generally characterized more by necrosis and serous or hemorrhagic exudation and infiltration than by the breaking down of tissue and frank pus-production. In this the organism is in marked contrast to the staphylococcus pyogenes aureus, which practically always produces dissolution of tissue and pus. Moreover, the streptococcus inflammations are more commonly accompanied by lymphangitis than are those due to the staphylococcus pyogenes aureus.

In a few instances we have met with a streptococcus whose colonies assume a well-marked yellow color on blood-serum, but which in other respects are like the long-chained forms above described.

Diagnosis.—The streptococcus pyogenes may often be identified by the cover-glass examination alone through its characteristic chain-formation, but this may not be apparent and the result of cultures must then be awaited.

Cultures from the blood during life should be made in ascites-dextrose bouillon (see page 135).

For cultures from joints in acute rheumatism, E. C. Rosenow recommends aspirating the joint with a sterile syringe, and mixing 1 c.c. or less of the joint fluid with fluid ascites-

dextrose agar, cooled to 40° C. After the tubes have been inoculated and their contents mixed, they are to be placed in cold water to solidify the agar and then in the incubator. In this way the streptococci are given a chance to grow without oxygen in the depths of the medium, a condition which favors their growth.

The ascites-dextrose agar tubes are prepared as follows: 2 per cent. nutrient agar containing 0.2 to 1 per cent. of dextrose, of a reaction 0.4 to 0.6 per cent. acid to phenolphthalein, is boiled in tubes containing 7 to 8 c.c. each for a few minutes to expel absorbed oxygen, cooled to 50° C., and then to each tube is added 2 or 3 c.c. of sterile ascitic fluid which has been previously heated to 60° C. for twenty-four hours. The tubes are then cooled to 40° C. and inoculated as above described.

In erysipelas the streptococcus is most readily found in the extreme margin of the affected area where the process is newest. The skin should be cleansed with soap and water, and with alcohol. Then with a sterile knife-point or a large needle a small wound should be made, and some of the blood and exudate pressed out from the tissue beneath. From this cultures and cover-glasses may be prepared.

Pneumococcus.—*Synonyms:* Diplococcus pneumoniæ; Micrococcus lanceolatus; Micrococcus of sputum-septicemia; Micrococcus pneumoniæ crouposæ.

Blood-serum.—Minute colorless, transparent colonies, resembling very small drops of dew (Fig. 36).

Morphology.—Pairs of rather small oval, conical, or lancet-shaped organisms, the broader ends being in apposition. The organism varies somewhat in size, and one of the "pair" may be smaller than the other (Fig. 33). In some cases atypical or involution forms are seen, especially if the culture be more than twenty-four hours old. No capsules are ordinarily observed in cultures with ordinary methods of staining. In the "water of condensation" of the blood-serum tube, chains may be formed resembling those of the streptococcus, but differing from the chains of that organism by the oval or lancet form of the elements of which they are composed.

In pus, blood, or in other material the organism is invested with a hyaline zone, called the capsule (see Figs. 34, 35). This is composed of a mucin-like substance. It may be

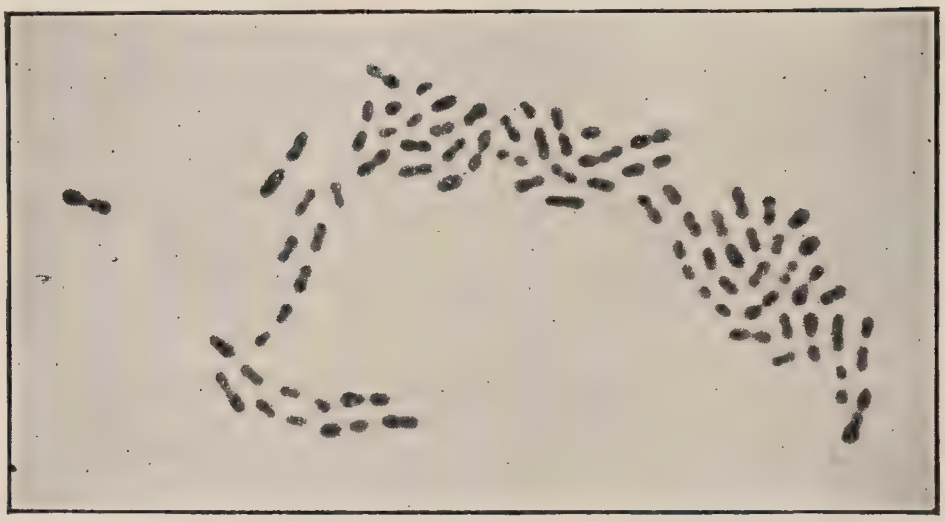


FIG. 33.—Pneumococci from a culture; × 2000 (Wright and Brown).

seen usually in cover-glass preparations stained by the ordinary methods, especially if the preparations be examined in water-mounting.

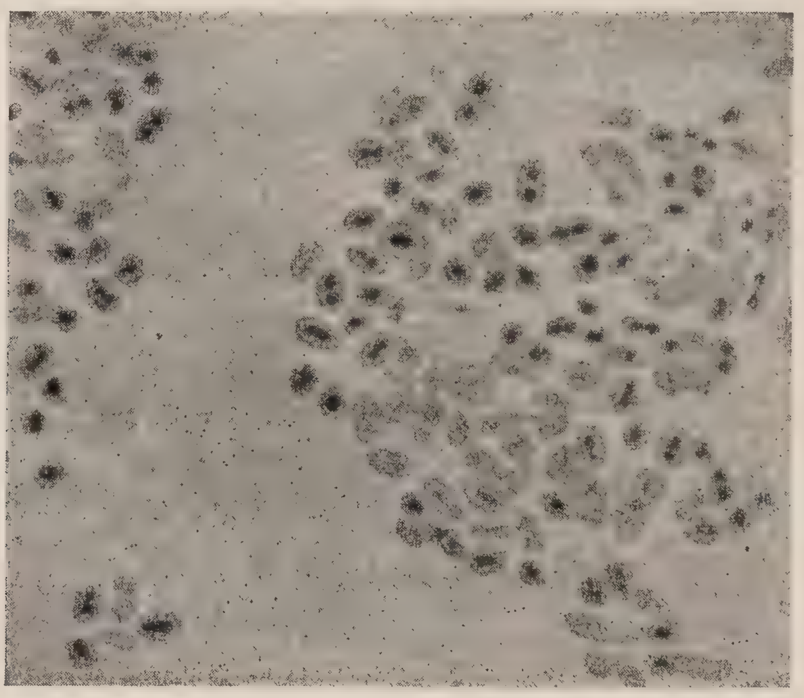


FIG. 34.—Pneumococci with capsules in a cover-glass preparation from pericardial exudate stained by W. H. Smith's method; × 1500 (W. H. Smith; photo. by L. S. Brown).

Stained by Gram's method. Not motile.

Glycerin Agar-agar.—Feeble growth of very minute grayish colonies.

Bouillon.—Clouded faintly.

Litmus Milk.—Sometimes turned pink and coagulated. Growth on other culture-media is very feeble. The organism dies out rapidly in cultures. To keep it viable it should be transplanted every forty-eight hours.

Pathogenesis.—The pneumococcus is very pathogenic for mice and rabbits, less so for guinea-pigs.

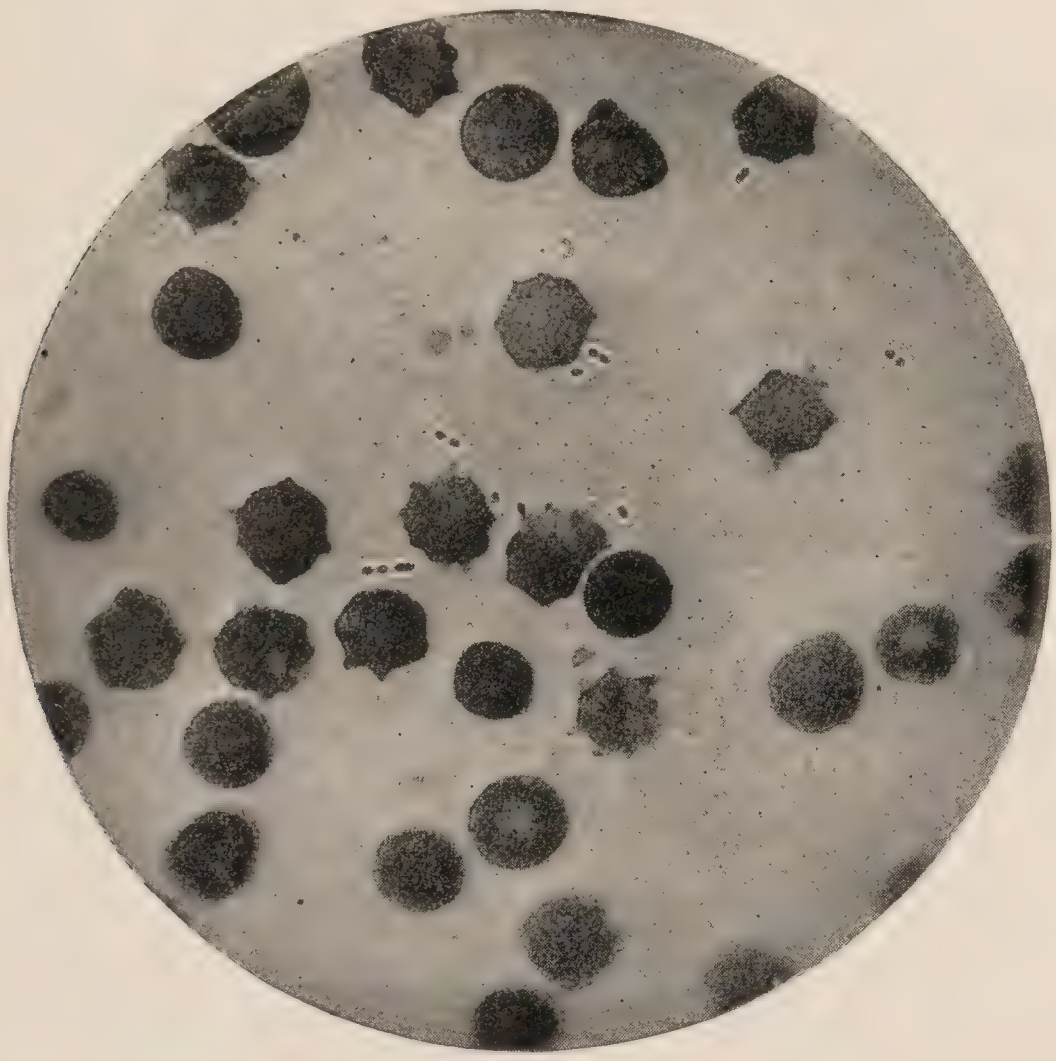


FIG. 35.—Diplococcus pneumoniæ; cover-glass preparation from the heart's blood of a rabbit; × 1000 (Fränkel and Pfeiffer).

Subcutaneous inoculation with virulent cultures causes the death of mice in from twenty-four to thirty-six hours, and of rabbits in from thirty-six to forty-eight hours, with septicemia.

This infection is the "sputum-septicemia" of Sternberg. At the autopsy there will be found in the blood everywhere the characteristic encapsulated lancet-shaped organisms, usually in pairs (Fig. 35). Great variation in the virulence of the organism is observed. In some cases no effect will be produced by the inoculation; in others a more or less exten-

sive fibrino-purulent exudation will be produced about the point of inoculation, and the animal will survive for a considerable length of time or recover. Inoculation into the ear-vein or peritoneal cavity of a rabbit will sometimes cause a rapidly fatal septicemia, when subcutaneous inoculation with the same culture will only cause a local reaction. The virulence of the pneumococcus is quickly lessened by cultivation.

Occurrence.—The pneumococcus may be demonstrated in the pulmonary exudate of practically all cases of genuine lobar or croupous pneumonia. At autopsies on cases of this disease it may be found in large numbers in the consolidated lung, and sometimes in smaller numbers in the blood of other internal organs. Cultures from the lung may sometimes show the presence of other bacteria in addition to the pneumococcus, but these are to be regarded as either secondary infections or contaminations from the smaller bronchi.

The pneumococci in the pneumonic exudate die in large numbers after a time, and in cases near resolution numerous capsules may be found in cover-glass preparations from the lung in which it is impossible to demonstrate the organism by staining methods.

The pneumococcus is also frequently found in bronchopneumonia, acute peri- and endo-carditis, acute pleuritis and empyema, acute purulent meningitis, and in otitis media. In cases of pneumonia and bronchitis it may be present in the sputum in large numbers. It has been observed in cases of peritonitis, of synovitis, of osteomyelitis, and of abscess-formation in various situations.

At autopsies on individuals dead of these conditions it may be frequently found, by means of cultures and animal inoculations, generally distributed throughout the internal organs in variable numbers. It is also often present in the mouth and in the saliva of healthy individuals.

Diagnosis.—If the pneumococcus be present in very small numbers in pathological material, the quickest and most certain method of demonstrating its presence is the inoculation of a mouse with some of the material (see page 121).

This is also the best way to prove the identity of the organism. In severe infections it may be demonstrated during life in the blood. Ascitic dextrose bouillon should be the medium employed for cultures.

The pneumococcus can usually be identified in exudates, blood, tissues, or sputum by examination of cover-glass preparations alone, by reason of its peculiar morphology and

FIG. 36.—Streptococcus capsulatus in cover-glass preparation from sputum stained by W. H. Smith's method (W. H. Smith; photo. by L. S. Brown).

its possession of a capsule. The capsule can be seen in most instances in cover-glass preparations, stained in the usual manner, if they be examined in water-mount. The capsules appear as a hyaline material usually with definite outlines surrounding the paired organisms. If it be desired to stain the capsules, W. H. Smith's method should be used.

Streptococcus Capsulatus.—This seems to be the best name to apply to a capsule-bearing bacterium resembling

both the pneumococcus and the streptococcus pyogenes in morphology, but differing definitely from them in cultural and other peculiarities. It has been found chiefly in lobar pneumonia, but occurs in other inflammatory processes and probably has been sometimes mistaken for the pneumococcus or the streptococcus pyogenes. The lack of general recognition of this micro-organism as a separate and distinct species vitiates, to some extent, the value of statistical

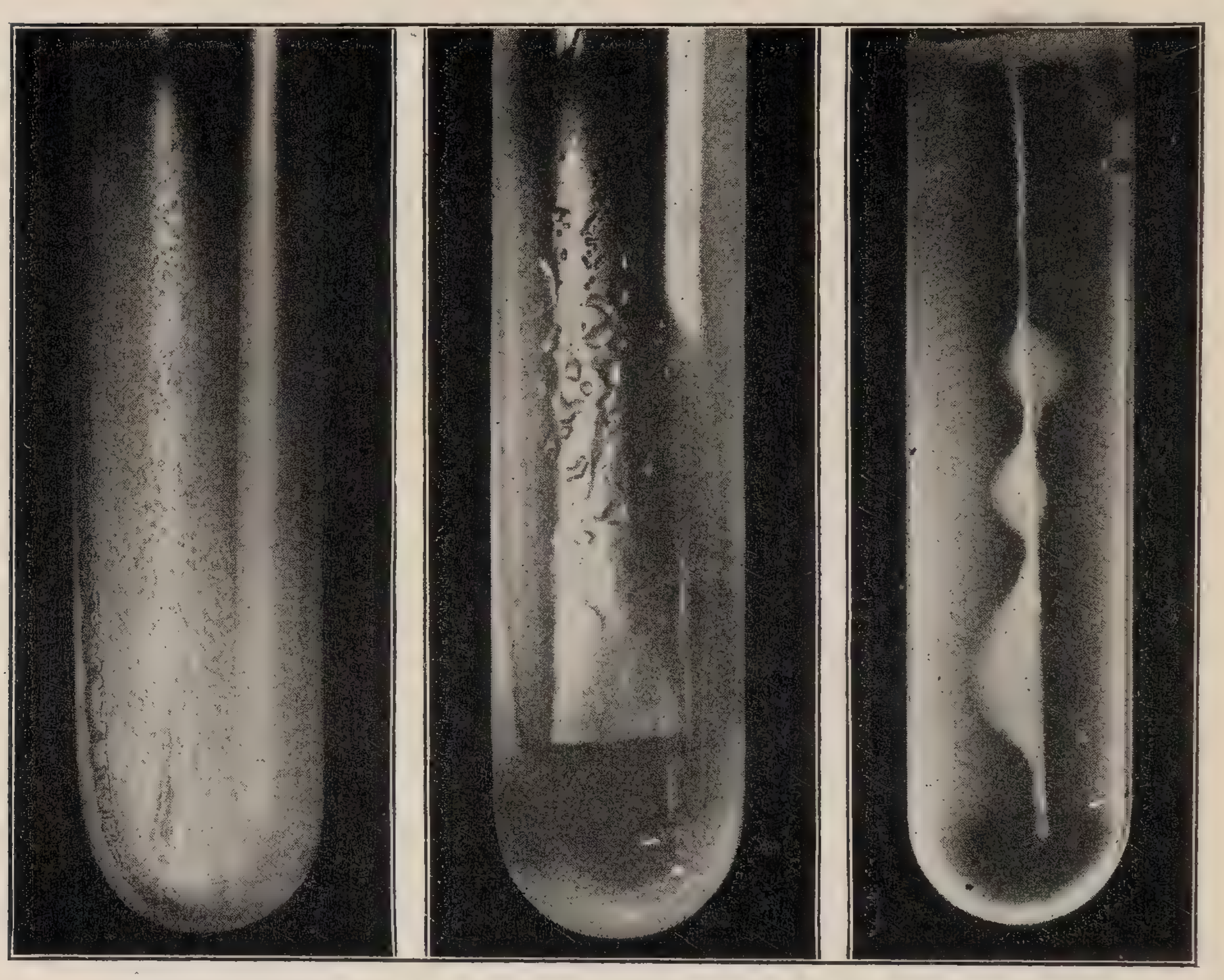


FIG. 37. FIG. 38. FIG. 39.

FIG. 37.—Pneumococcus; blood-serum culture.

FIG. 38.—Streptococcus capsulatus; blood-serum culture.

FIG. 39.—Streptococcus capsulatus; glucose-agar stab culture (Oscar Richardson; photos. by L. S. Brown).

studies of infections with the streptococcus and the pneumococcus.

The best description of the streptococcus capsulatus is that of Oscar Richardson, based upon observations on 4 cases of lobar pneumonia in the laboratory of the Massachusetts General Hospital. Richardson points out the fol-

lowing chief characteristics by which it may be distinguished from the pneumococcus and the streptococcus pyogenes:

1. The capsules persist in cultures (see Fig. 40).

2. On the surface of coagulated blood-serum its colonies are flat, colorless, viscid, mucus-like, of irregular outline, and may attain a diameter of 2 or 3 mm. They may become confluent and form large patches of mucus-like material (see Fig. 38).

3. In glucose-agar stab, adjusted to a reaction of 0.5, there is growth all along the line of inoculation, from which, in

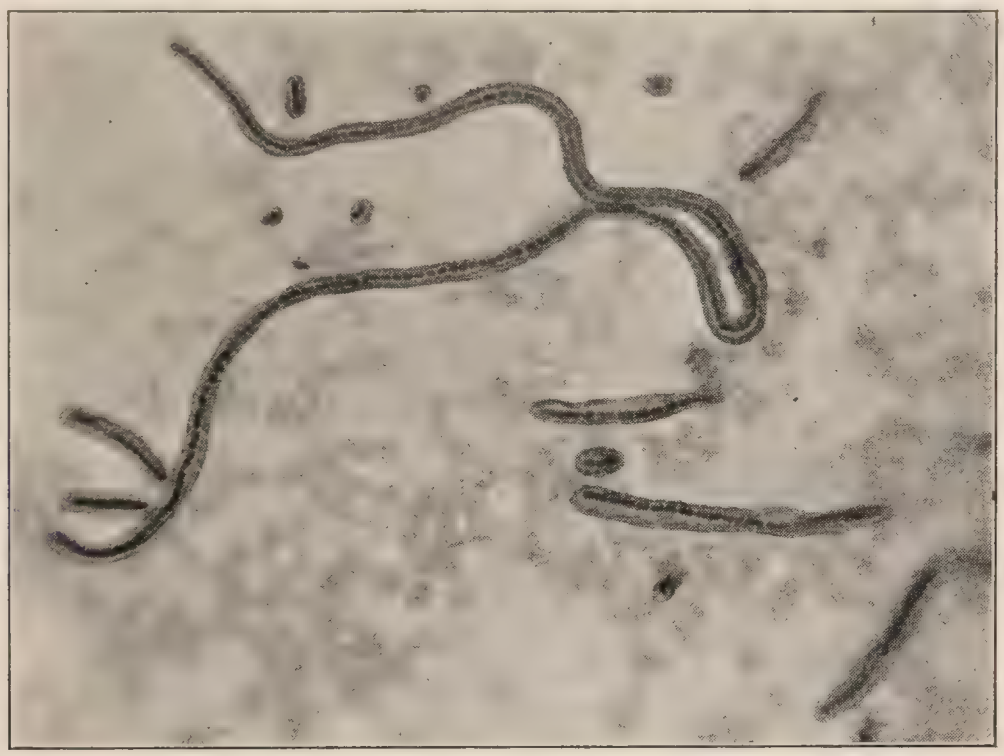


FIG. 40.—Streptococcus capsulatus, from a blood culture in a case of pneumonia; stained by W. H. Smith's method; × about 700 (W. H. Smith; photo by L. S. Brown).

places, fusiform or hemispherical masses of growth extend into the surrounding medium in a vertical plane, apparently occupying clefts in the medium (Fig. 39). It is very important for the development of these characteristic appearances that the glucose-agar be known to have at the time of inoculation a reaction very close to that above indicated.

Gonococcus.—*Morphology.*—Cocci of medium size, composed usually of two hemispheres separated by a narrow unstained interval. Sometimes two of these pairs of hemispheres are joined together in the manner of "tetrads," or

10

groups of four, showing evidence that division occurs in two directions at right angles to each other (Fig. 41). Decolorized by Gram's method.

The gonococcus will not grow satisfactorily upon any of the culture-media ordinarily employed, but requires special media for its cultivation.

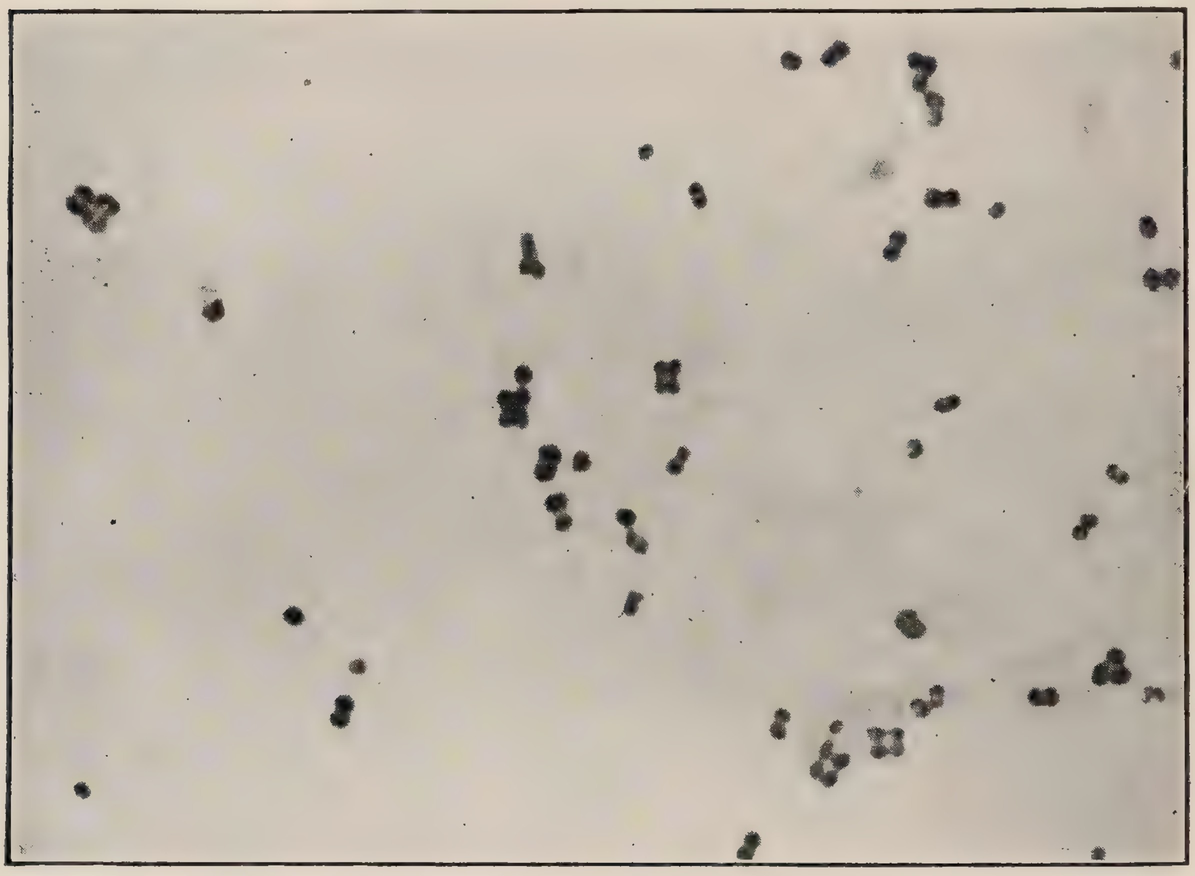


FIG. 41.—Gonococci from a culture, showing formation of tetrads and variability in the size of the cocci; $\times$ 2000 (Wright and Brown).

The *colonies* on suitable culture-media appear after eighteen to twenty-four hours as minute, grayish, translucent points. Later they may attain a diameter of 2 mm. Under low magnifying power a well-developed colony is seen to consist of a generally circular expansion, with thin, translucent, sharply defined margins, becoming brownish, granular, and denser toward the center, which is made up of coarse brownish clumps closely packed together (Fig. 42).

Special Culture-media.—The essential constituent of culture-media upon which the gonococcus will grow seems to be the blood-serum or similar albuminous fluid from the animal body.

Probably the most convenient culture-medium for the cultivation of the gonococcus is *hydrocele-fluid agar.* This medium consists of sterile hydrocele fluid mixed with fluid agar-agar at a temperature of 40° C., in the proportion of 1 part of hydrocele fluid to 2 or 3 parts of agar-agar. The hydrocele fluid is to be obtained under the strictest precautions to avoid contamination with bacteria, thoroughly sterilized vessels, etc., being used.

Ordinary tubes of plain agar-agar (2 per cent.) which have been previously sterilized in the usual manner are melted and

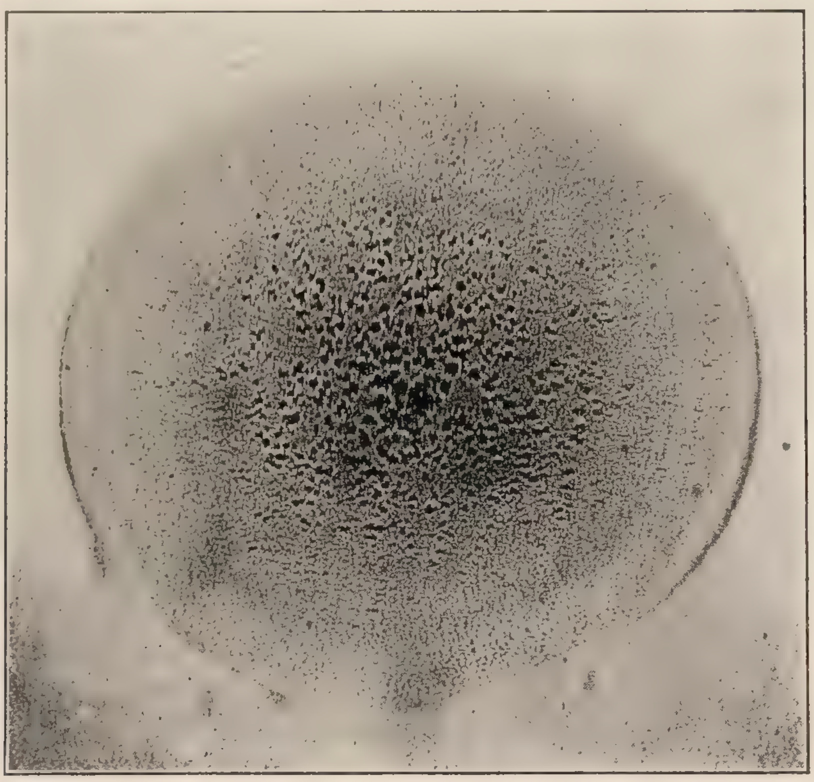


FIG. 42.—Gonococcus colony (low magnifying power; photo by L. S. Brown).

brought to a temperature of 40° C. in a water-bath. To the fluid agar-agar in each tube the sterile hydrocele fluid is then added in the proportion of one-third to one-half the volume of the agar-agar, care being taken to avoid contamination. For the transfer of the serum to the agar-agar tubes a sterilized pipette may be used. The tubes may then be infected and their contents poured into sterilized Petri dishes, as in the plate method previously described (see page 113), or the tubes may be placed on their sides in a slightly inclined position and the agar-agar allowed to solidify, thus forming "slants" which may be kept on hand ready for use. In order to test for the presence of contaminating bacteria in

these slants, it is well to place them in the incubator for twenty-four hours after they have become solid, so that any bacteria which may be present in them will form colonies and manifest themselves. Some pathological fluids which are rich in albumin, such as the serous exudate of pleuritis, may be used in the place of the hydrocele fluid as above described.

Occurrence.—The presence of the gonococcus may be demonstrated in the pus of acute gonorrhea and gonorrheal ophthalmia. It occurs also in a certain proportion of cases of purulent salpingitis. It has been found in peritonitis, endocarditis, pericarditis, myocarditis, pleuritis, and arthritis, as well as in peri-urethral abscess, in abscess of the glands of Bartholini, and in other acute inflammatory processes. In a few cases of endocarditis it has been demonstrated in the blood during life.

Diagnosis.—For practical purposes the gonococcus may be sufficiently identified in pus by cover-glass examination of the same. Cocci in the form of paired hemispheres chiefly situated within the pus-cells and decolorizing by Gram's method of staining may be regarded as gonococci with a fair degree of certainty.[1] The fact that they decolorize by Gram's method serves to distinguish them from the pyogenic staphylococci and streptococci, for these may also be present inside leucocytes, and may in some instances look like gonococci. The identification by this means is not beyond question. To make it more certain the isolation and study of the suspected cocci in cultures are necessary. In cultures, not only should the organism show the peculiarities of morphology, of staining, and of colony growth above described, but it should be rigidly determined that it does not grow on ordinary agar-agar.

In making transplants to plain agar avoid carrying over any of the albuminous material of the special culture-

[1] There is no trustworthy evidence that any other Gram-decolorizing micrococcus than the gonococcus ever occurs in gonorrheal pus. F. T. Lord, working in the laboratory of the Massachusetts General Hospital, examined by cultures the pus from 22 cases of gonorrhea, and in none could he find any Gram-decolorizing micrococcus other than the gonococcus.

medium, for this material may permit some growth of the gonococcus on the plain agar.

In applying the test of decolorization by Gram's method, colonies not more than forty-eight hours old should be used, because Gram-staining cocci in older cultures may be more or less decolorized by this method. In proof of the necessity of cultures for confirming the identity of the gonococcus in certain instances we may state that we have met with a Gram-decolorizing coccus in an arthritis of the knee, clini-

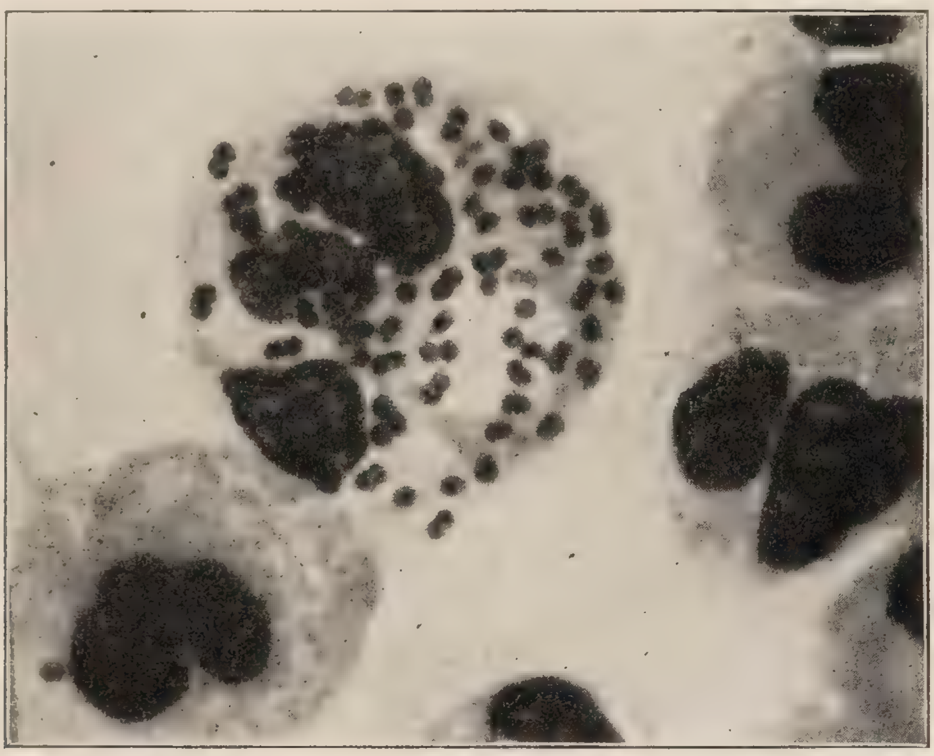


FIG. 43.—Gonococci inside a leucocyte. Cover-glass preparation from gonorrheal pus; × 2000 (Wright and Brown).

cally of gonorrheal origin, which, in cover-glass preparations from the exudate, was regarded as the gonococcus, but which was found not to be that organism by the study of it in cultures.

If it is desired to obtain cultures of the gonococcus from the pus of gonorrheal urethritis, the case should not be more than of a few days' duration, because cases of longer duration will usually show the presence of other bacteria whose colonies overgrow the feebly growing colonies of the gonococcus. An organism which may be mistaken for the gonococcus is a coccus growing in large milk-white colonies on all media, but staining by Gram's method. This coccus is

frequently found in gonorrheal pus after the discharge has lasted several days. Other cocci also occur.

The pus for culture purposes may be collected on a "swab," and the special culture-medium directly infected with this. The gonococcus retains its vitality in the pus on the swab for a number of hours, but care should be taken to avoid drying.

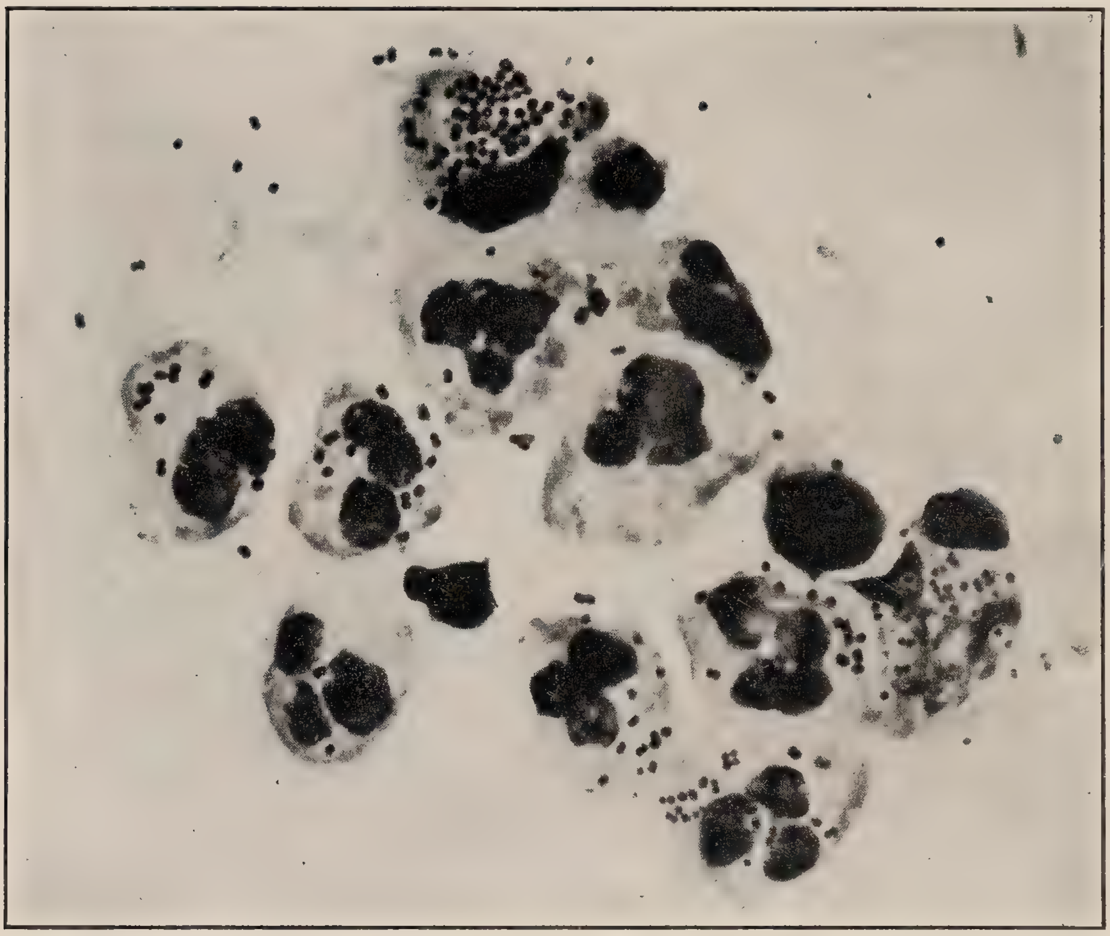


FIG. 44.—Micrococcus catarrhalis in smear from sputum (F. T. Lord; photo by L. S. Brown).

In a certain proportion of cases of purulent inflammation of the oviducts gonococci may be found and cultivated, as above indicated. The majority of cases, however, will have sterile pus, while in a small percentage of cases the ordinary pyogenic cocci will be present.

Cultures from the blood in cases of gonorrheal endocarditis should be made in hydrocele bouillon. This consists of 1 part of sterile hydrocele fluid and 3 parts of sterile bouillon mixed under aseptic precautions.

Method of Staining for Gonococci.—Prepare a cover-glass with the pus, spreading it thinly with the platinum

wire. The practice of spreading a small drop of pus between two cover-glasses and drawing them apart is objectionable. After "fixing," stain the preparation by the following method:

1. Stain with aniline-gentian-violet solution for thirty seconds, without heating.
2. Wash in water.
3. Cover the preparation with Gram's iodin solution for thirty seconds.
4. Wash in water.
5. Wash with alcohol (95 per cent.) until the color ceases to come out of the preparation.
6. Wash in water.
7. Stain in saturated aqueous solution of Bismarck brown for thirty seconds.
8. Wash in water and mount.

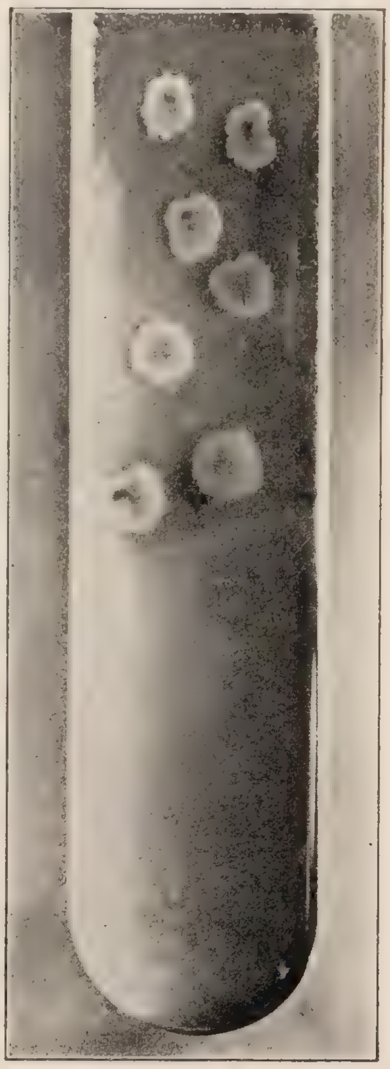


FIG. 45.—Micrococcus catarrhalis colonies on agar (F. T. Lord; photo by L. S. Brown).

This method is nothing but Gram's method and after-staining with Bismarck brown. With it the gonococci are stained brown, and other pyogenic cocci stained blue-black.

W. F. Whitney has suggested the use of a 1 per cent. aqueous solution of pyronin in place of the solution of Bismarck brown in step 7. The gonococci are stained red by the pyronin.

Micrococcus Catarrhalis.—This micrococcus may be found in the sputum in inflammatory conditions of the respiratory tract and cannot be distinguished in its morphology and staining reactions from the gonococcus or from the Diplococcus intracellularis meningitidis (Fig. 47). The appearances of its colonies on ordinary culture-media are, however, characteristic. They are large, white, of irregular outline, and have elevated central portions. They are friable, not viscid, and grow readily at room-temperature (Fig. 45).

Micrococcus Tetragenus.—The *colonies* are small, white, and elevated. Growth is slow.

Morphology.—Micrococci arranged in fours, or "tetrads," held together by a gelatinous substance (Fig. 46).

Stained by Gram's method. Not motile.

Gelatin Stab.—Feeble growth in the form of minute spherical masses along the line of stab with a small white, slightly elevated point at the surface of the medium. The gelatin is not liquefied.

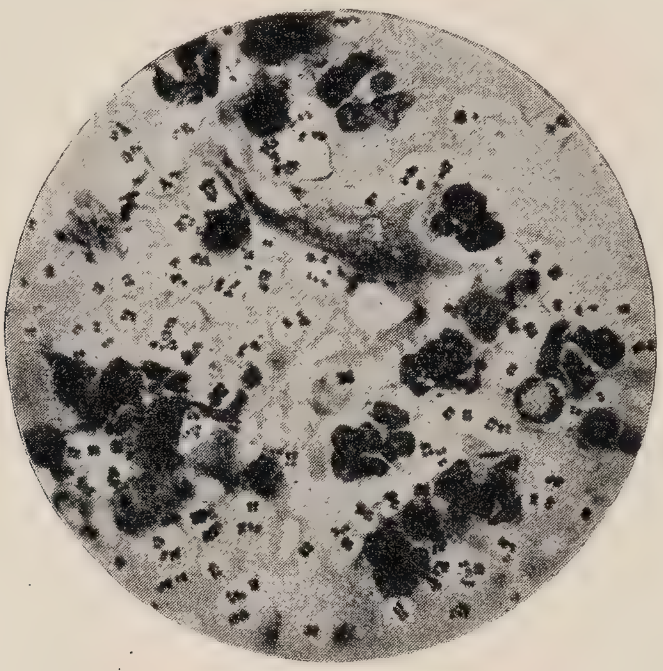


FIG. 46.—Micrococcus tetragenus in pus from a white mouse; × 615 (Heim).

Agar-agar Slant.—Moist, glistening, grayish-white translucent streak with wavy margins.

Potato.—Growth is in the form of a thick, irregular, slimy-looking patch. The growth on agar-agar and on potato may be drawn into thin threads by the platinum wire.

Pathogenesis.—Subcutaneous inoculation of mice and guinea-pigs may lead to a fatal septicemia or only a local pus-formation. Intravenous or intraperitoneal inoculation of rabbits may also produce septicemia and death.

At autopsy the micrococci, arranged in tetrads, are found in the blood generally, but most numerously in the spleen. They can readily be demonstrated by cover-glass preparations.

Occurrence.—" Found not infrequently in phthisical cavities and sputum, occasionally in association with pyogenic

cocci in abscesses connected with carious teeth and about the neck and jaws and middle ear, rarely in abscesses elsewhere. It has been considered to be non-pathogenic for man, but it has been found in pure culture in closed abscesses in man, and Viquerst has proved experimentally that it is capable of causing suppuration in human beings" (Welch).

Diplococcus Intracellularis Meningitidis.—*Morphology.*—Diplococci, occurring as paired hemispheres, separated by a well-marked unstained interval and showing considerable variation in size in cultures (Fig. 47). The

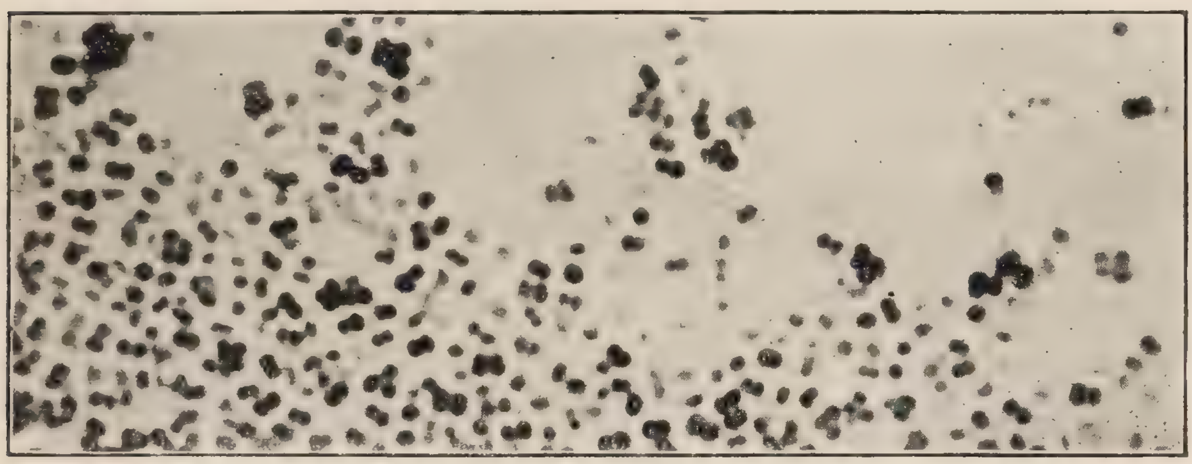


FIG. 47.—Diplococcus intracellularis meningitidis from a culture, showing the tendency toward grouping in fours or tetrads; $\times$ 2000 (Wright and Brown).

larger forms are regarded as involution or degenerate forms. The organism shows a tendency to grouping in fours or tetrads.

In cover-glass preparations from the meningeal exudate the diplococcus frequently is situated inside leucocytes, and sometimes within the nucleus (Fig. 48). The appearances are very much like those of gonorrheal pus. It is decolorized by Gram's method.

Blood-serum.—The colonies appear after about twenty-four hours, and after forty-eight hours may attain a diameter of 2 or 3 mm. They are round, colorless, shining, slightly convex or flat, moist, and viscid-looking. They may become confluent.

Agar-agar.—Round, flat, grayish, translucent, moist, shining colonies, attaining a diameter of 2 or 3 mm. after twenty-

four hours in the incubator. They may become confluent, and in a "slant" culture the growth appears as a grayish, translucent, moist, shining streak about 3 mm. in width,

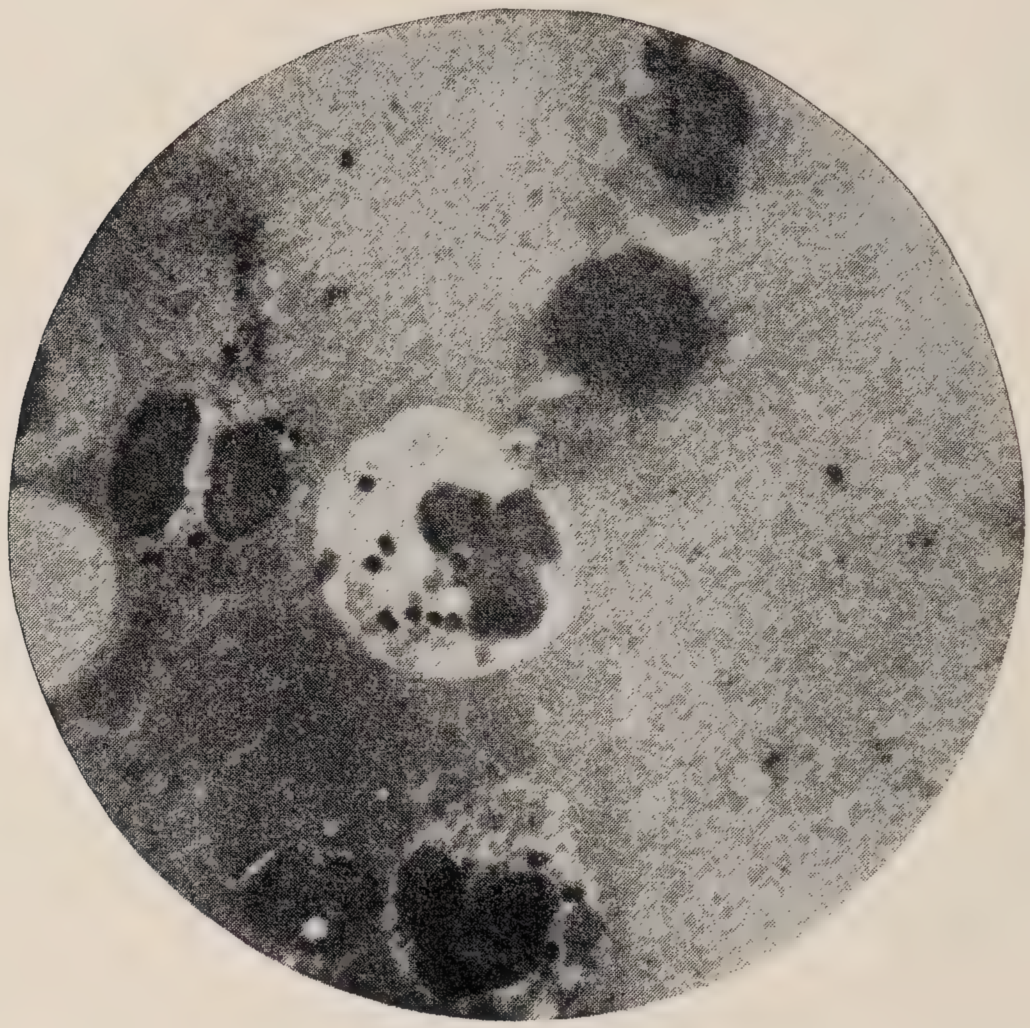


FIG. 48.—Diplococcus intracellularis meningitidis in polynuclear leucocytes of meningeal exudate (Jaeger).

with smooth margins. Under a low magnifying power the colonies are homogeneous, semi-translucent, and not granular.

Sugar-agar Stab-culture.—Feeble growth not extending all along the line of inoculation.

Bouillon.—Medium slightly to moderately clouded. At the bottom of the tube a whitish sediment, which may rise as a viscid string when the tube is shaken.

Potato.—Very feeble or doubtful growth, giving the surface of the potato a moist appearance at the most.

Litmus-milk.—Growth without visible change in the medium.

Gelatin.—Feeble growth. No liquefaction.

Vitality.—The organism quickly dies out under cultivation. It seems to survive somewhat better on blood-serum than on agar-agar, but cultures on the former only seventy-two hours old may be found no longer capable of growth after transplantation.

Pathogenesis.—Intraperitoneal inoculation of guinea-pigs and rabbits gives very uncertain results. Mice are said to succumb to subcutaneous inoculation, with some invasion of the blood by the organism.

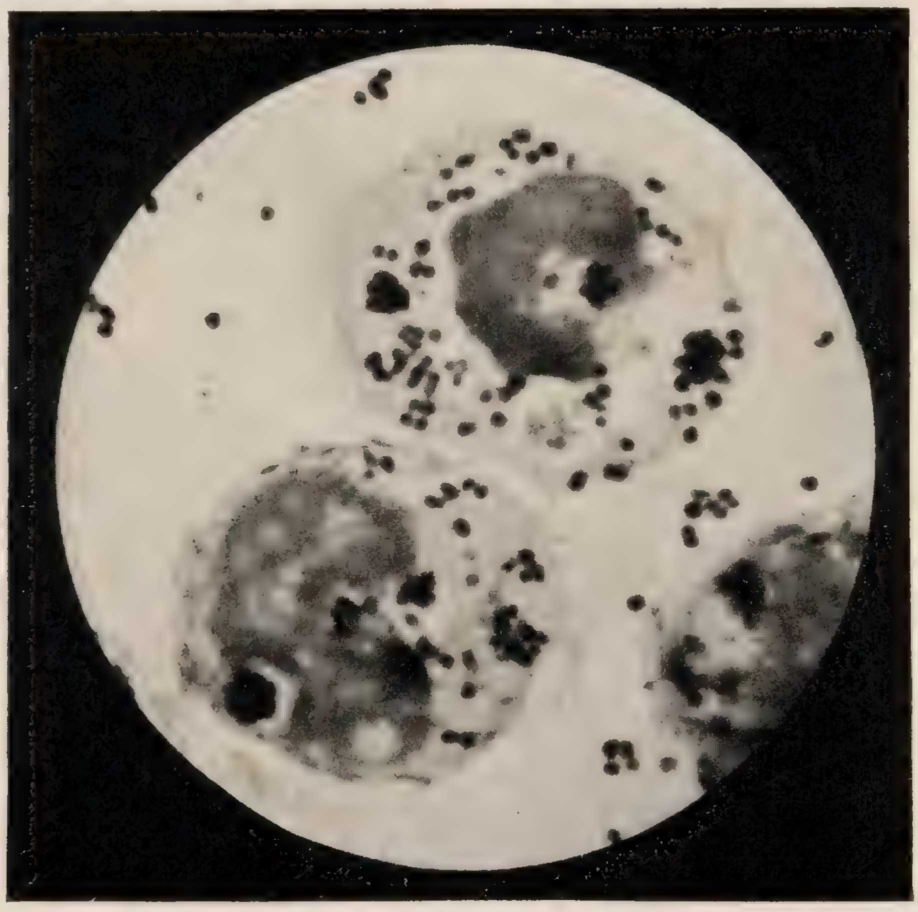


FIG. 49.—Diplococcus intracellularis meningitidis in leucocytes. Cover-glass preparation from peritoneal exudate in a guinea-pig; × 2000 (Wright and Brown).

Exceptionally, we have found that the intraperitoneal injection of a bouillon suspension of a twenty-four-hour blood-serum culture in the quantity of about 1 c.c. would kill guinea-pigs within forty-eight hours.

At the autopsy there is an accumulation of a cloudy or blood-stained fluid in the peritoneal cavity, some little enlargement of the spleen, and some injection of the peritoneum. Microscopical examination of the exudate shows numerous leucocytes crowded with the diplococci (Fig. 49).

The culture-test gives no evidence of general infection of the blood.

Occurrence.—Found in the meningeal exudate of certain cases of epidemic cerebro-spinal meningitis. It is situated mainly inside the pus-cells, some of which may contain many diplococci. In some cases the presence of the organism in the exudate may be difficult or impossible to demonstrate, and it is probable that it rapidly dies out. It has been found in the arthritis and in the pneumonia which sometimes accompanies the disease, and in the nasal secretion both of sick and of well individuals. A general invasion of the circulation by the micro-organism does not occur as a rule.

Diagnosis (see also section on Lumbar Puncture).—In exudates suspected of containing it, cover-glass preparations should be stained by the method for gonococci (see p. 150). The presence of micrococci, often in the pus-cells, decolorized by Gram's method, is sufficient for its identification in the meningeal exudate as far as our present knowledge goes. Cultures are necessary if the material is from other sources than the meninges.

The material for examination is best obtained with the platinum loop from the superior portions of the nasal cavities.

Bacillus Diphtheriæ.—*Blood-serum.*—Round, elevated, smooth colonies of the color of the medium. They may attain a diameter of 2 mm. after forty-eight hours in the incubator.

Morphology.—Bacilli varying markedly in size and shape, of irregular outline, and showing great variability in the staining of different parts of their protoplasm (Figs. 50, 51). The presence in a palely tinted rod of deeply stained granules and points, frequently situated at the extremities, and the occurrence of irregular forms, often club-like in shape, with a constriction in the middle, are appearances which are very characteristic of the bacillus when grown upon blood-serum and stained with Löffler's methylene-blue solution. Its morphology and staining peculiarities are so characteristic when cultivated upon blood-serum that the microscopical examination is in most cases sufficient for its identification.

FIG. 50.—Diphtheria bacilli from a culture on blood-serum, stained by Löffler's methylene-blue solution, showing deeply stained points; $\times$ 2000 (Wright and Brown).

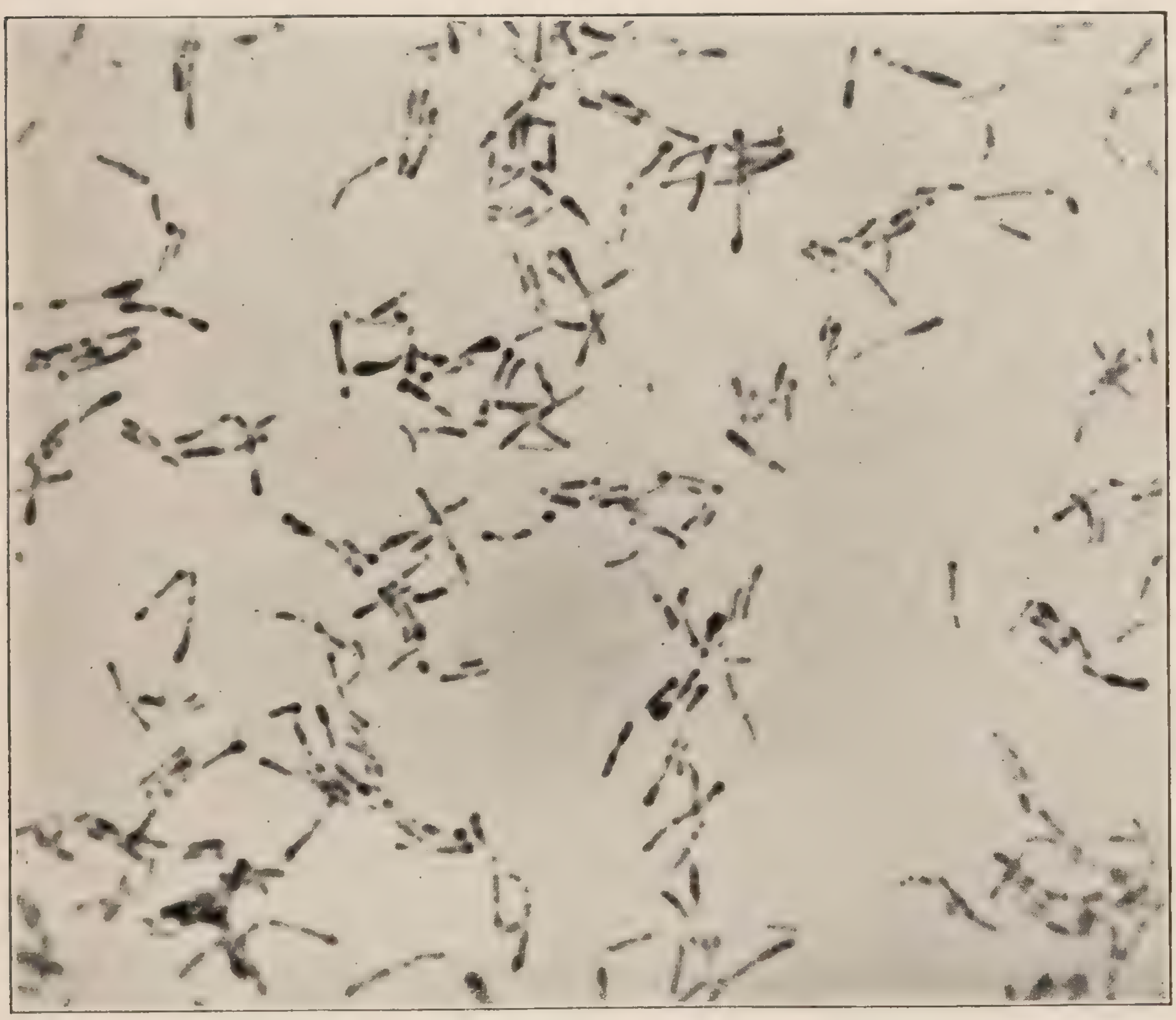


FIG. 51.—Diphtheria bacilli from a culture on blood-serum, stained by Löffler's methylene-blue solution, showing long and irregularly shaped forms of the bacillus, as well as the irregularity of staining; $\times$ 2000 (Wright and Brown).

When grown upon other culture-media than blood-serum, however, its morphology and staining peculiarities are not so characteristic, and they may vary markedly in different media.

Stained by Gram's method. Not motile.

Bouillon.—Grows usually in the form of fine grains at the bottom of the tube and adherent to the sides, the bouillon remaining clear or becoming slightly clouded. The reaction of the media rapidly becomes acid, but changes to alkaline after a variable length of time.

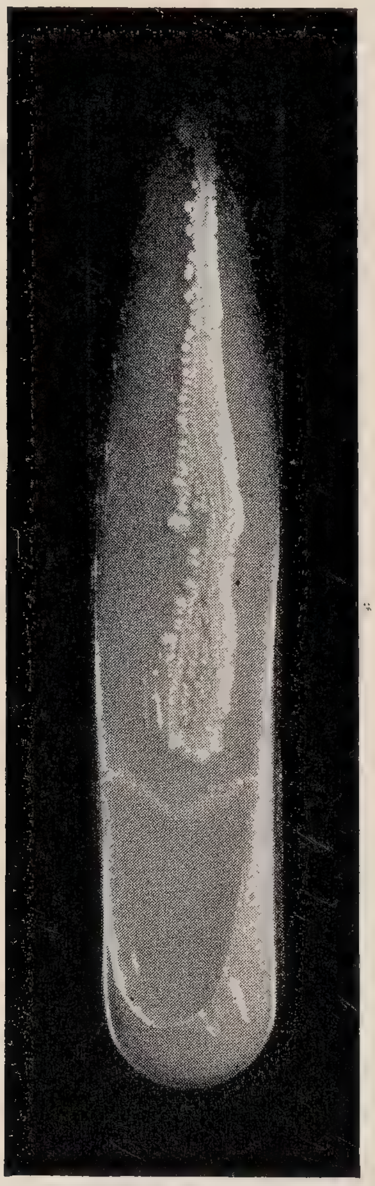


FIG. 52.—Bacillus diphtheriæ; agar-agar culture (photograph by Dr. Henry Koplik).

Potato.—Growth not visible to the naked eye. The bacillus grows, however, to a certain extent, and usually assumes very atypical and irregular forms (involution forms).

Agar-agar and Gelatin.—The growth on these media is slower and more feeble than upon blood-serum (Fig. 52). It presents nothing remarkable.

Pathogenesis.—Subcutaneous inoculations of guinea-pigs are fatal in thirty-six to seventy-two hours in the case of virulent cultures. The lesions produced consist usually of edema, hemorrhage, and fibrinopurulent exudation about the point of inoculation in the subcutaneous tissue, hemorrhagic enlargement of the lymphatic glands, congestion and edema of the lungs, hemorrhages into the suprarenal capsules, and less frequently necrosis of the liver and pleural effusions. Histological examination of the lymph-glands shows marked "fragmentation" of the nuclei of the cells, giving rise to numerous deeply staining globules of chromatin scattered throughout them. The bacilli are ordinarily found only about the point of inoculation, but cultures from the various

organs will sometimes show the presence of the bacilli in some of them.

Toxin-production.—The effects produced by infection with the bacillus diphtheriæ are due to the action of a so-called toxalbumin or "toxin" which the organism manufactures in its growth. The poisonous substance is produced in cultures. Its presence may be demonstrated by inoculating an animal with a small quantity of the filtrate, obtained by passing a bouillon culture some weeks old through an unglazed porcelain filtering apparatus, by which all the bacteria are removed from the fluid.

The "toxin" is contained in solution in the filtrate. If this be fairly rich in "toxin," the injection of $\frac{1}{10}$ c.c. subcutaneously into a guinea-pig should lead to the death of the animal in three or four days with the various lesions above described. The local reaction, however, is not so marked as in the case of inoculation with the bacilli. With the ordinary bouillon the production of a great amount of "toxin" by the growth of the diphtheria bacilli in it is very uncertain. Theobald Smith has recently shown that this uncertainty is due to the presence of variable amounts of muscle-sugar from the meat used in the preparation of the bouillon, and that this substance prevents the accumulation of toxin. He has found that that bouillon yields the most toxin which has the least muscle-sugar in it. He prepares such bouillon as follows: "Beef infusion, prepared either by extracting in the cold or at 60° C., is inoculated in the evening with a rich fluid culture of some acid-producing bacterium (I use temporarily B. coli) and placed in the thermostat. Early next morning the infusion, covered with a thin layer of broth, is boiled, filtered, pepton and salt added, and the neutralization and sterilization carried on as usual." This bouillon is placed in two 500 c.c. Erlenmeyer flasks, 250 c.c. in each flask. In these, cultures are made and kept for at least eight days in the incubator. After this time a fair amount of toxin may be assumed to have developed, and the contents of the flask are then filtered through a porcelain cylinder. A filtrate is to be regarded as containing a reasonable amount of toxin if $\frac{1}{10}$ c.c., injected subcutaneously, kills a medium-sized guinea-pig in three days. The filtrate containing the "toxin" can be preserved by the addition of 0.5 per cent. pure carbolic acid.

Occurrence.—The bacillus diphtheriæ occurs in the local lesions in all cases of true diphtheria, in rhinitis fibrinosa, and in many cases of the milder forms of acute inflammation of the air-passages. It may persist in the mucous membrane of the throat and nose long after convalescence has been established.

In fatal cases of diphtheria the organism is nearly always present in the lungs, and it may be often found by culture-methods more or less generally distributed in comparatively small numbers throughout the internal organs. In the majority of diphtheria autopsies an invasion of the blood-stream by the streptococcus pyogenes, and sometimes by other bacteria, may be demonstrated by cultures. The bacillus may also be found in company with other bacteria in ulcerated or excoriated surfaces on the skin, as well as in other suppurative processes, in individuals affected with diphtheria, and on the soiled linen of the patient. The infection of wounds with the bacillus diphtheriæ has also been observed without coincident diphtheria.

Diagnosis.—The bacteriological diagnosis of infection with the bacillus diphtheriæ depends upon the characteristic morphology and peculiarities of staining, as well as rapidity of growth, which this organism presents when cultivated upon coagulated blood-serum. The identification by direct cover-glass examination of the exudate is very uncertain.

The method is as follows: A blood-serum culture-tube is inoculated with a small amount of the material from the mucous membrane affected, and is placed in the incubator twelve to eighteen hours. After this length of time the resulting growth is examined by cover-glass preparations stained either with Löffler's methylene-blue solution or by one of the special methods given below.

The bacillus diphtheriæ, if present, may then be recognized and differentiated from other bacteria present in the preparation by its characteristic morphology and peculiarity of staining, described on page 156. The gross appearances of the culture present little that is characteristic, as a rule, and the main reliance is to be placed on the microscopic examination. Early in the infection the greater part of the growth may be made up of the specific bacilli, but toward convalescence they fall into the minority. The ordinary forms of agar-agar culture are not suitable for use in the bacteriological diagnosis of diphtheria, owing to the comparative feebleness of the growth of the organism on these

media, and because of the fact that its microscopic appearances when cultivated on such media are not sufficiently characteristic.

The material for culture is very conveniently obtained by means of sterilized cotton swabs. In collecting this material the swab is removed from its test-tube and touched to the affected areas of the mucous membrane of either the nose or throat. It is then to be gently rubbed over the surface of a blood-serum culture-tube, or it may be replaced in the test-tube and the inoculation of the culture-tube made later in the laboratory. In the latter case the inoculation should be made within an hour or two after the material has been collected, the infected swab meanwhile being prevented from drying by firmly replacing the cotton plug.

In cases with membrane-formation the greatest number of bacilli are on the surface or in the upper layer of the membrane, and the swab should therefore be touched to these portions rather than to the tissue beneath.

Special Methods of Staining the Bacillus Diphtheriæ.—Owing to the fact that the bacillus diphtheriæ may be recognized by its peculiar morphology and characteristic staining in cover-glass preparations from its growth upon certain culture-media, as already pointed out, various special staining methods have been devised for accentuating and rendering more striking to the eye the peculiar deeply stained points and granules in the bodies of the individual bacilli, which have been referred to as of great importance in the identification of the organism.

These special methods of staining are said to be of great advantage in cases where only a few specific bacilli may be suspected to be present among a large number of other bacteria.

Neisser's Method.—1. Stain for one to three seconds in a solution which is made as follows: 1 gram of methylene-blue (Gruebler), in powder, is dissolved in 20 c.c. of 96 per cent. alcohol. To this add 950 c.c. of distilled water and 50 c.c. of glacial acetic acid, and filter.

2. Wash in water.

3. Stain for three to five seconds in a solution of vesuvin (Bismarck brown), made by dissolving 2 grams of the dye (in powder) in 1000 c.c. of boiling distilled water.

4. Wash in water, and mount.

The diphtheria bacilli stained by this method appear as pale brown rods bearing bluish-black granules, usually of oval shape and of a diameter somewhat greater than the rod. The majority

of the bacilli show a granule at each end or at only one end, but not rarely three granules are present, one being near the middle of the rod. More granules than these are exceptional (see Fig. 53).

The bacilli must have been grown on Löffler's blood-serum medium, coagulated at 100° C., and the culture must be at least nine hours and not more than twenty-four hours old.

Hunt's Method.—1. Stain in saturated aqueous solution of methylene-blue one minute without heating.

2. Wash in water.

3. Cover with aqueous solution of tannic acid, 10 per cent., for ten seconds.

4. Wash in water.

5. Stain in saturated aqueous solution of methyl-orange one minute, without heating.

6. Wash in water.

7. Dry, and mount in balsam.

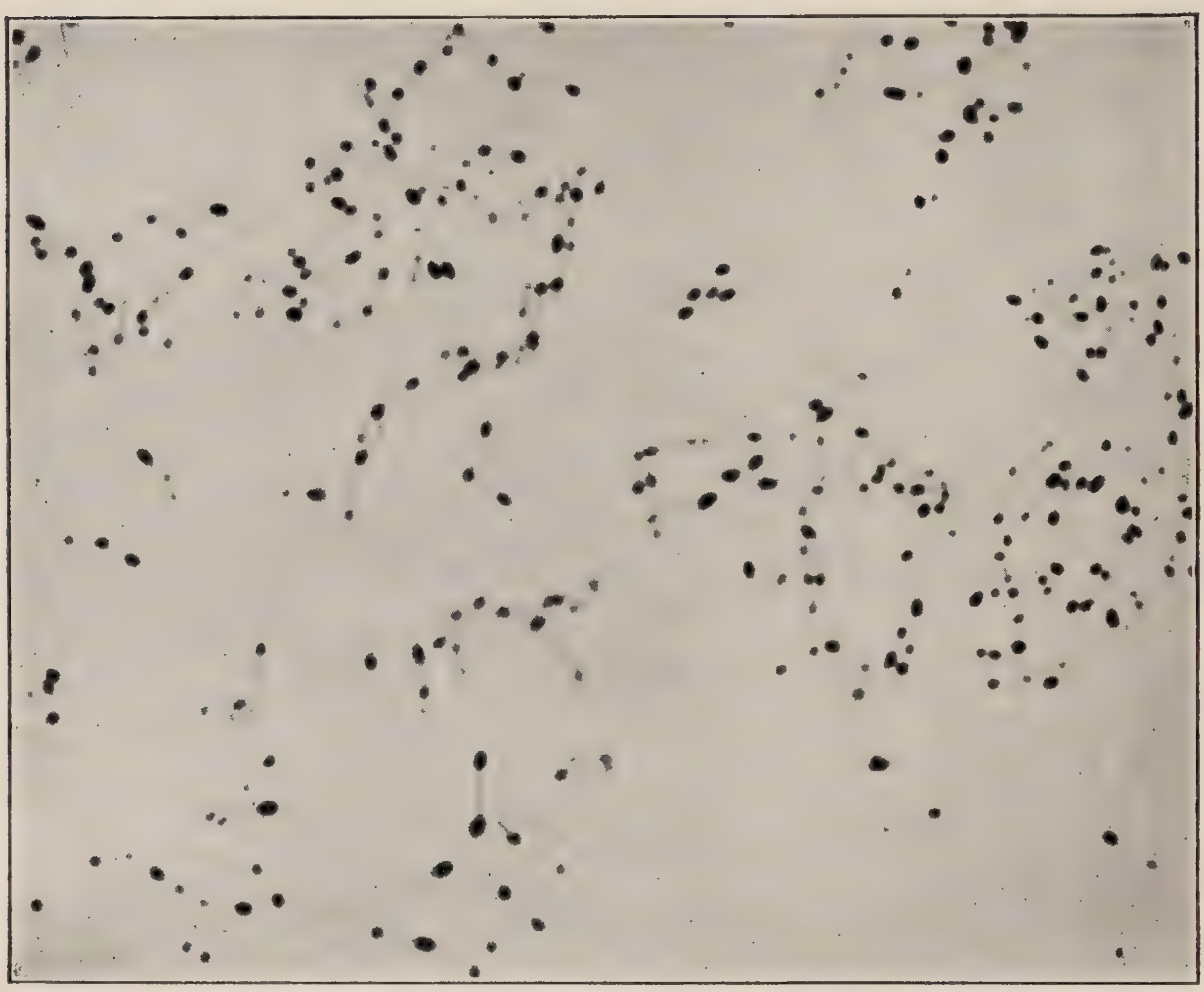


FIG. 53.—Diphtheria bacilli from blood-serum culture stained according to Neisser's method; × 2000 (Wright and Brown).

By this method the granules, etc., are dark blue or almost black, and stand out very sharply against the light-green coloring of the body of the bacillus (see Fig. 54). The solution of methyl-orange should be freshly prepared, for it deteriorates in a few days.

Bacillus of Typhoid Fever.—*Synonyms:* Bacillus typhi abdominalis; Bacillus typhosus; Typhoid bacillus.

Blood-serum.—Round, grayish, viscid-looking colonies, which may attain a diameter of 2 mm. after forty-eight hours in the incubator.

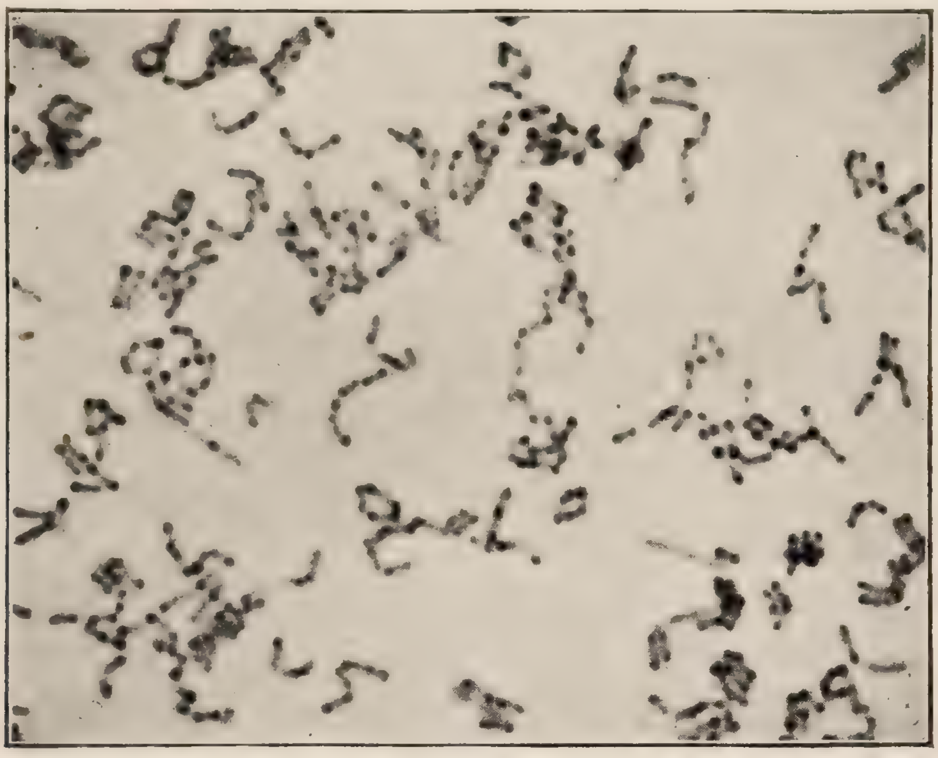


FIG. 54.—Diphtheria bacilli from blood-serum culture stained according to Hunt's method; × 2000 (Wright and Brown).

Morphology.—Medium-sized bacilli with rounded ends, generally short (Fig. 55), but sometimes long or thread-like,

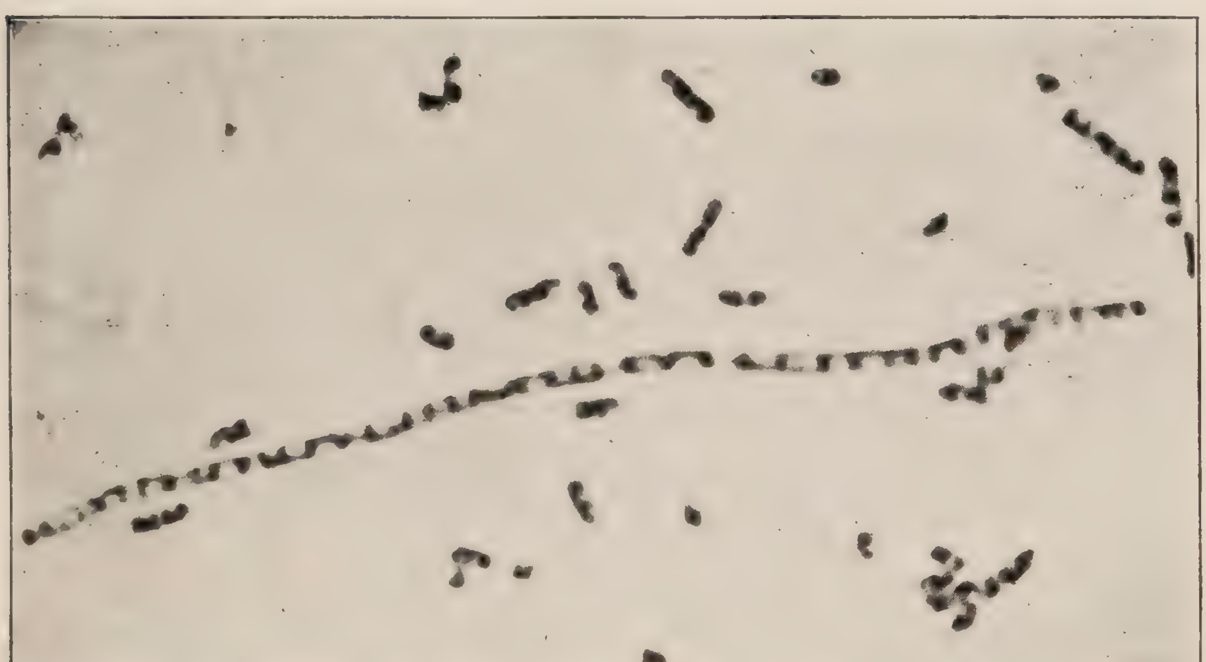


FIG. 55.—Typhoid bacilli from a bouillon culture, showing characteristic irregularity in staining and variability in length; × 2000 (Wright and Brown).

and frequently showing faintly-stained, sharply-defined areas in their protoplasm (Figs. 55 and 56).

Gelatin Slant.—Broad translucent streak with wavy, irregular margins. The gelatin is not liquefied. Growth is

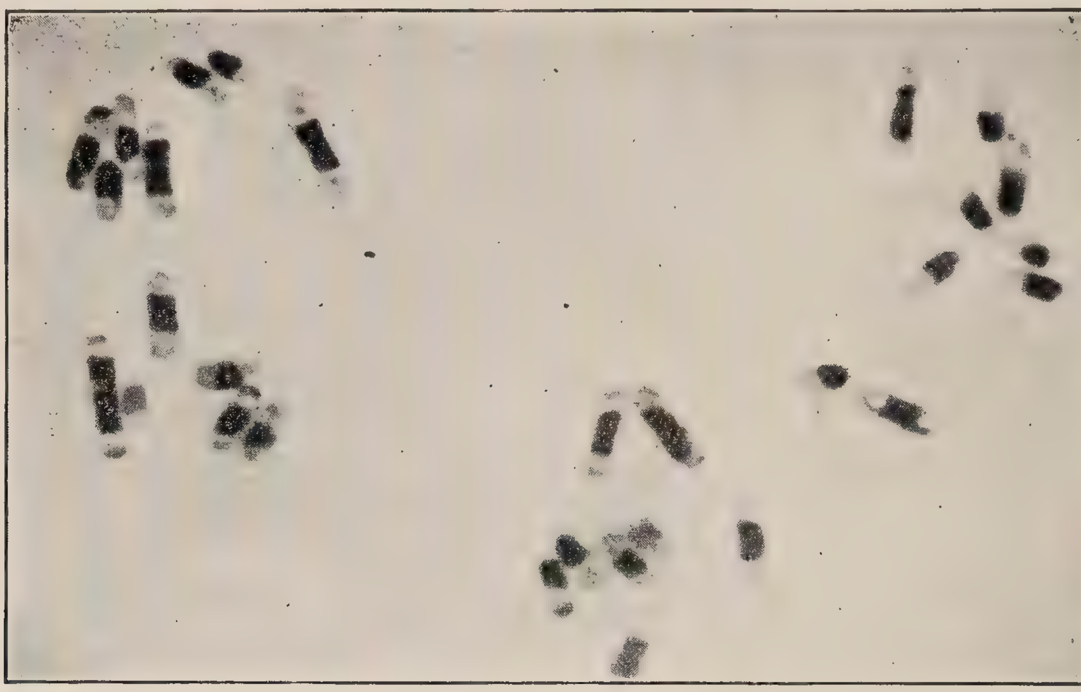


FIG. 56.—Typhoid bacilli from a culture on potato, showing unstained areas in the bacilli and polar granules; × 2000 (Wright and Brown).

slower than that of the bacillus coli communis in the same medium.

An isolated colony, slightly magnified, on gelatin, is shown in Fig. 57.

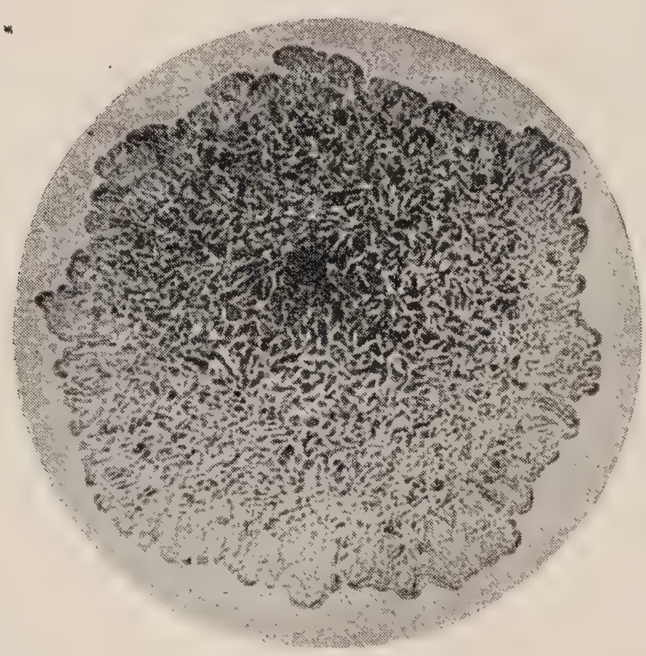


FIG. 57.—Bacillus of typhoid fever: superficial colony two days old, as seen upon the surface of a gelatin plate; × 20 (Heim).

Glucose-gelatin Stab.—Growth all along the line of inoculation in the form of confluent spherical grayish colonies, and on the surface about the point of entrance of the platinum

wire in the form of a circular translucent grayish layer. No production of gas-bubbles. No liquefaction.

Glucose Agar-agar.—Growth similar to that in the preceding. No gas-formation.

Litmus-milk.—No visible change.

Potato.—Growth occurs, but it is usually invisible.

FIG. 58.—Typhoid bacilli, from a culture on agar-agar, showing flagella, from a preparation stained by Dr. Hugh Williams; × 2000 (Wright and Brown).

Dunham's Peptone Solution.—No indol-production—*i. e.* no red color appearing in the twenty-four- to forty-eight-hour cultures after the addition of 5 drops of concentrated sulphuric acid, c. p., and 1 cubic centimeter of a solution of sodium nitrite, 1 : 10,000.

Motility.—Very marked.

Flagella.—(Fig. 58) may be demonstrated by the special methods of staining described elsewhere.

Decolorized by Gram's method. Does not form spores.

Bouillon.—Clouded, with the formation of some sediment. The clouding of the medium is not so marked as in the case of the bacillus coli communis. In general, the growth of the typhoid organism is not so vigorous on culture-media as is the growth of the bacillus coli communis.

When to a bouillon culture a small quantity of the blood-serum of a typhoid-fever patient is added, the bacilli lose their motility and aggregate in clumps ("serum reaction").

Pathogenesis.—The inoculation of animals is usually without results if moderate quantities of the organism are used. Sometimes, however, death occurs apparently from the effects of the toxic material injected.

Occurrence.—Found in the spleen in large numbers at autopsies in typhoid fever. Its presence may also be demonstrated in the intestinal lesions, rose spots, mesenteric lymph-glands, liver, bile, kidneys, urine, and blood of the heart. As a rule, the number of bacilli found in the liver, kidneys, and blood of the heart is small. In the bile they may be numerous and may persist in it for a long period of time after the disease has subsided. In some cases the urine contains enormous numbers of the bacilli.

The typhoid bacillus may also occur in the suppurative sequelæ of typhoid fever, especially those involving bones. In these conditions, however, it may be accompanied by the pyogenic cocci. Occurs in contaminated water.

Paratyphoid Bacilli.—These bacilli have been found associated with inflammatory processes and with fevers clinically resembling typhoid fever. They differ from the typhoid bacillus chiefly in two respects: they produce gas in glucose media and show different agglutination reactions. Two types of them, known as types "A" and "B," are generally recognized. Type A behaves in all other respects essentially like the typhoid bacillus. Type B does not coagulate milk, but makes it alkaline and, after ten days or more, translucent.

Its colonies are generally larger, less translucent, and may have a porcelain white color. Its growth on potato may appear as a thick brownish layer.

Differential Diagnosis between the Bacillus of Typhoid Fever and the Bacillus Coli Communis.—The most important points of difference between these two organisms are as follows, and to distinguish with certainty between them it is necessary that attention be paid to all of them:

Motility.—The typhoid bacillus is actively motile, the bacillus coli communis not motile or exceptionally motile.

Potato Cultures.—The typhoid bacillus usually grows invisibly, the bacillus coli communis as a dirty, slimy layer.

Gas-production in Media Containing Glucose.—The bacillus coli communis produces gas, the typhoid bacillus does not.

Litmus-milk Cultures.—The bacillus coli communis changes the blue color of the medium to a pink color and usually coagulates the milk. The typhoid bacillus produces no visible change.

Indol-production.—The bacillus coli communis produces indol, the typhoid bacillus does not.

Serum or Clump Reaction.—The typhoid bacillus shows the clump reaction, while the bacillus coli communis does not. As it is not always possible to have a typhoid serum at hand by which to determine whether this reaction is present, a stock of dried blood from a typhoid case, contained in filter-paper, may be kept ready for use. That this is quite practicable has been clearly shown by Dr. Mark W. Richardson. The blood may be obtained from the heart at the autopsy of a typhoid-fever case by soaking a piece of filter-paper with it. This is allowed to dry, and then is cut into pieces about 1 cm. square. When it is desired to make the test, one of these pieces is extracted with ten or fifteen drops of water, and a drop of this extract is mixed with a drop of an eighteen- to twenty-four-hour bouillon culture on a slide, covered with a cover-glass, and examined with the high-power dry lens. Dr. Richardson has found that

the blood under these conditions retains for months its "clumping" power with reference to the typhoid bacillus.

Other differences are—the production of a red color in litmus-lactose agar-agar by the bacillus coli communis, and no change in color of this medium by the typhoid bacillus, and the slower and less vigorous growth of the typhoid bacillus in culture-media.

The Blood-serum Reaction in Typhoid Fever.—A few drops of the blood of a suspected case of typhoid fever are collected in a small test-tube, either from the finger or the ear. After clotting has taken place, transfer a drop of the serum by means of a medicine-dropper to forty drops of a recent bouillon culture of the typhoid bacillus. After mixing, place a drop of the mixture on a slide, cover it

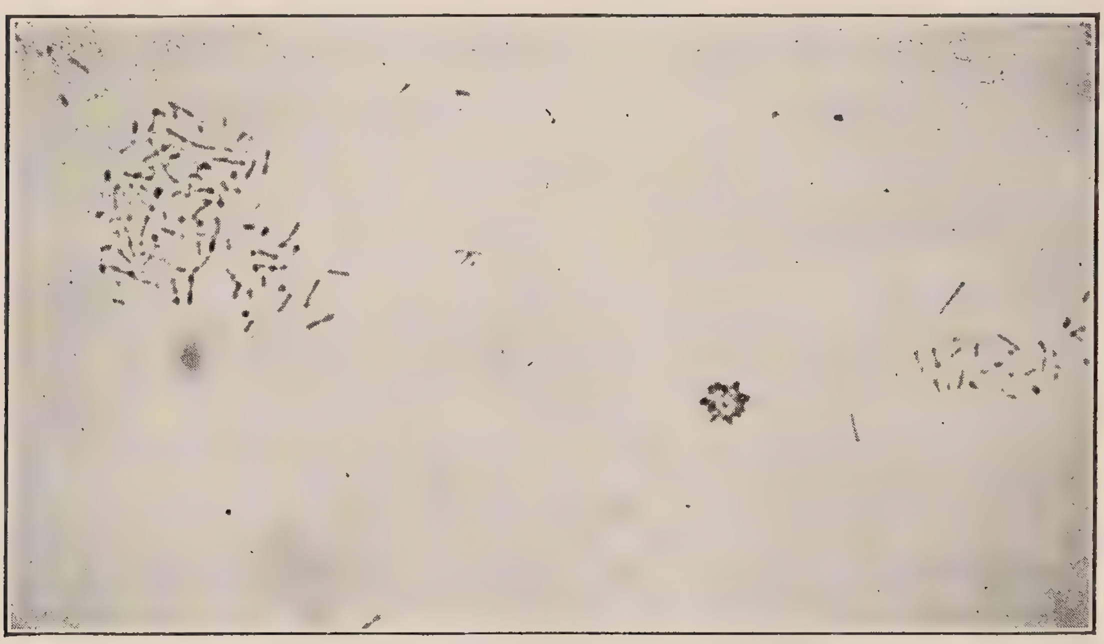


FIG. 59.—Showing the clumping of typhoid bacilli in the serum-reaction. Wet preparation, not stained. At one point a crenated red blood-corpuscle is seen (Wright and Brown).

with a cover-glass, and examine with a high-power dry objective.

If the bacilli are seen to be motionless and to be agglomerated in clumps within twenty minutes, the reaction is to be regarded as present and typical. If the clumping occurs within thirty minutes, but free bacilli are still moving, the reaction is to be regarded as doubtful. The reaction is present, as a rule, only after the first week of typhoid fever.

The slide and cover-glass should be sterilized in a flame after the test is completed. The bouillon culture used should be grown at room-temperature and should not be more than four or five days old. Before carrying out the test it is well to assure one's self that the bacteria are actively motile by examining a drop of the culture as above indicated. The stock cultures of the typhoid bacilli are best made on agar-agar.

The reaction may also be obtained from the dried blood. A few drops of the blood may be collected on a glass slide or a piece of paper and allowed to dry. It may then be brought to the laboratory, where as much of the dried blood as would correspond to a drop is scraped from the slide into a small test-tube, containing forty drops of a bouillon-culture of the typhoid bacillus, or as much of the paper as may be assumed to contain one drop of blood is placed in forty drops of the bouillon culture and allowed to soak for a few minutes therein. Microscopical examination is then made with the mixture thus obtained as above indicated.

Cultivation of the Typhoid Bacillus from the Blood during Life.—One method of doing this is indicated on page 100. It is important that the blood should be diluted with a large excess of sterile bouillon—say, 1 or 2 c.c. to 100 or 200 c.c. of bouillon in flasks.

The Ox-bile Method.—Test-tubes containing 5 c.c. of plain ox-bile sterilized by steam are used. If a precipitate appears on heating, it may be filtered off before using without detriment to the medium. The blood of the patient is added to the bile in the test-tube up to 2.5 c.c. in amount. It may be obtained from one of the superficial veins of the forearm by means of a syringe, or by pricking the lobe of the ear with a lance-pointed knife and squeezing out drop by drop into the tube of bile. The mixture of bile and blood is then incubated at 37° C. for twelve to fifteen hours, when transfers of a few loopsful are made either to a v. Drigalski-Conradi plate or other culture-medium in order to prepare to establish the identity of the bacteria that may have developed.

Cultivation of the Typhoid Bacillus from the Feces.[1] —Various culture-media have been devised upon which colonies of typhoid bacilli will grow with appearances distinguishing them from colonies of colon bacilli and other bacteria. Of these, we here describe but three. In all cases the colonies supposed to be those of the typhoid bacillus should be proved by transplants in various media and the bacteria shown to conform to the requirements indicated on pages 167 and 168.

Make an emulsion of one or more loopsful of the feces in sterile bouillon and from this make five or six plate-cultures with the medium, using one to five loopsful of the emulsion to each plate. The plates are to be examined after twelve to eighteen hours in the incubator.

The colonies of typhoid bacilli below the surface of the medium are of irregular shape and present filamentous or thread-like processes extending into the medium. The colonies of colon bacilli beneath the surface of the medium are larger, denser, and darker, and do not present the filamentous processes. The surface colonies of the typhoid bacillus are smaller, less dense, thinner, and more homogeneous than are the colonies of colon bacilli.

Medium of v. Drigalski and Conradi.—(*a*) Preparation of agar: 1 kilo of finely ground beef is mixed with two liters of water and allowed to stand until the next day. The resulting meat infusion is boiled for an hour, filtered, and to it are added 20 gm. of peptone (Witte), 20 gm. neutrose, 10 gm. sodium chlorid, and the whole boiled for another hour and filtered. To the filtrate 60 gm. of agar-agar are added and the mixture boiled for three hours (or one hour in the autoclave), is made faintly alkaline to litmus, is again filtered, and is boiled one-half hour more.

(*b*) Litmus solution: litmus solution (according to Kubel and Tiemann), 260 c.c., boil ten minutes, add 30 gm. chemically pure lactose, and boil for fifteen minutes.

(*c*) Add the hot litmus-lactose solution to the fluid agar.

[1] We are indebted to Dr. F. W. Peabody for advice in regard to choice of methods in this and the following paragraphs on the typhoid bacillus.

Shake well and readjust, if necessary, to a weak alkaline reaction. Next add 4 c.c. of a hot sterile solution of 10 per cent. anhydrous sodium carbonate and 20 c.c. of a freshly prepared solution of 0.1 gm. of crystal violet (B. Höchst) in 100 c.c. of warm sterile distilled water.

This medium should be stored in flasks of about 200 c.c. capacity in order to avoid protracted heating when it is desired to melt it. When used, the medium is poured into round glass plates 15 to 20 cm. in diameter in sufficient amount to form a layer at least 2 mm. thick and is allowed to solidify. Its surface is then inoculated with a suspension of the fecal material, applied by means of a glass rod bent in a certain form. Obvious means are to be taken to secure such a distribution of the bacteria that a good separation of the colonies will result. After inoculation the medium is to remain uncovered in the plates until the surface is dry.

After twenty-four hours in the incubator the colonies of the typhoid bacillus are blue in color and smaller than the colonies of colon bacilli, which are red and opaque. Colonies of other bacteria may be blue, but they are generally larger.

Medium of Endo.—To 1 liter of water are added 500 gm. of finely ground beef, 10 gm. of peptone, 5 gm. of sodium chlorid, and 30 gm. of agar. The mixture is thoroughly boiled, filtered, neutralized, and then made alkaline by the addition of 10 c.c. of a 10 per cent. solution of sodium carbonate. After this 10 gm. of chemically pure lactose is added and 5 c.c. of an alcoholic solution of fuchsin to color the medium red; then 25 c.c. of a 10 per cent. solution of sodium sulphite is added, and the medium begins to lose its color, but does not become completely colorless until the agar becomes solidified. The medium is placed in test-tubes, 15 c.c. to each tube, and sterilized by steam for thirty minutes.

The following points are important:

The lactose must be chemically pure; the sodium sulphite solution must be freshly prepared; the alcoholic solution of

fuchsin must be filtered. The medium must be kept in the dark or it will take on a red color. When the medium is to be used it is melted and poured into sterile Petri dishes, which are left uncovered until the agar is solid. The cultures are made in the manner indicated for the medium of v. Drigalski and Conradi.

The colonies of the colon bacillus on this medium are red, while those of the typhoid bacillus are colorless.

Malachite-green Media.—These media consist essentially of ordinary agar, gelatin, or bouillon, to which is added a certain amount of malachite green, which is an acid dye, having the property, in correct dilution, of hindering the growth of colon bacilli without affecting the growth of typhoid bacilli.

The preparation of these media is complicated by the fact that the different specimens of malachite-green dyes upon the market vary greatly in their inhibiting action upon the growth of the colon bacillus, and it is necessary to determine for each specimen of the dye the proportions in which it must be used to give the desired results. These proportions vary from 1 : 750 to 1 : 10,000. It has been recently stated that the "malachite-grün cryst. chem. rein" from the Höchst Dye Works is a chemically pure and constant product.

Plate-cultures with these media are to be prepared in the same manner as indicated for the medium of v. Drigalski and Conradi.

Bacillus Coli Communis.—*Synonyms:* Bacterium coli commune; Colon bacillus.

Blood-serum.—Rounded, grayish-white, slightly elevated, viscid-looking colonies, which may attain a diameter of 3 mm. after twenty-four hours in the incubator.

Morphology.—A medium-sized bacillus with rounded ends, often short or even coccus-like, but may grow in long forms. Faintly staining, sharply defined areas are present in the protoplasm (Fig. 60).

Gelatin Slant.—Grayish translucent strip with wavy mar-

gins. The gelatin is not liquefied. Growth is more rapid than in the case of the typhoid bacillus.

FIG. 60.—Bacilli coli communis from a bouillon culture, showing the irregularity of staining of the bacillus; $\times$ 2000 (Wright and Brown).

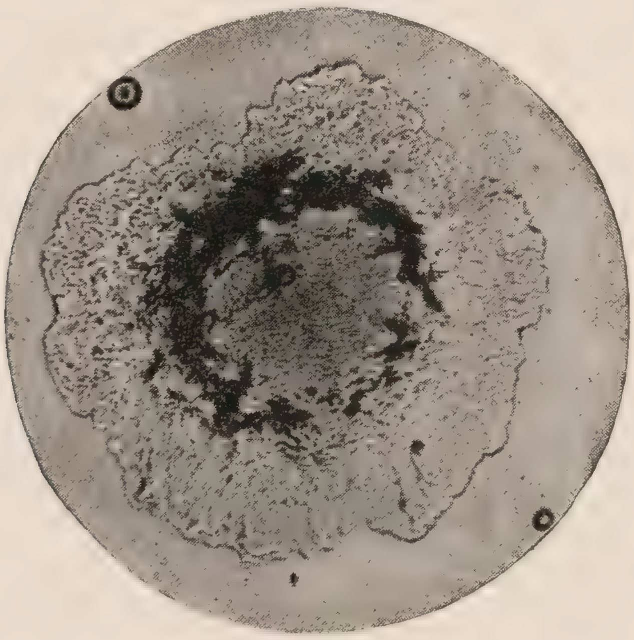


FIG. 61.—Bacillus coli communis: superficial colony two days old upon a gelatin plate; $\times$ 21 (Heim).

A single colony on a gelatin plate is shown in Fig. 61.

Glucose-gelatin Stab.—Growth along the line of stab in the form of confluent spherical colonies, and on the surface

about the point of entrance of the needle as a thin gray circular layer. Gas-bubbles are produced in the gelatin from fermentation of the glucose. The gelatin is not liquefied.

Glucose-agar-agar Stab.—Growth essentially the same as in the preceding, except that the gas-formation is more marked.

Litmus-milk.—Turned pink and usually coagulated.

Potato.—Dirty grayish or brownish, viscid-looking layer.

Dunham's Peptone Solution.—Marked indol-production. This is shown by the appearance of a red color in the culture after the addition of 5 drops of concentrated sulphuric acid, c. p., and 1 c.c. of a 1 : 10,000 solution of sodium nitrite. The culture in the peptone solution should have been at least twenty-four hours—or, better, forty-eight hours—in the incubator before the test is made.

Motility.—Usually not motile, but some varieties show sluggish independent movement.

Flagella.—May be demonstrated by the special methods of staining. They are less numerous than in the case of the typhoid bacillus.

Decolorized by Gram's method.

Bouillon.—Markedly clouded, with formation of a sediment. The clouding is more marked than in the case of the typhoid bacillus.

Lactose-litmus-agar-agar Slant.—Growth has a pink color, and the blue color of the medium is changed to red.

Pathogenesis.—"Its virulence as tested upon animals is variable, but is generally manifest only after inoculation of large doses, which kill by intoxication rather than infection" (Welch).

The lesions produced are not sufficiently characteristic to be detailed here.

Occurrence.—Occurs constantly in the intestinal canal, and is widely distributed in the external world.

"The colon bacillus is a frequent invader of the internal organs in all sorts of diseases, especially when there are in-

testinal lesions. It manifests no evident pathogenic action in most of these cases, and is then without clinical significance. It occurs frequently associated with other bacteria in infected wounds and other inflammations of exposed surfaces. Here also it does not usually appear to cause serious disturbance. The fact that the colon bacillus is so common and widely distributed, and found so often as a harmless invader, should lead to much caution in interpreting the significance of its presence when it occurs in definite lesions. There is no doubt, however, that it may be pathogenic for man. It plays an important rôle in inflammations of the urinary tract and biliary passages; also, but usually with less independence, in peritonitis and appendicitis.

"The list of diseases in which it may be found is a very long one, and includes inflammations in all organs and parts of the body. In general its pathogenic properties are of a mild character. One of its leading rôles is to invade territory already occupied by other bacteria or previously damaged. It may be concerned in the production of gall-stones, in the interior of which it has been found by the writer with great frequency" (Welch, Dennis's *System of Surgery*, vol. i.).

The bacillus above described is to be regarded as a type of a group of bacilli constituting the so-called "colon group" of bacilli. These present certain quantitative differences among themselves which are not quite sufficient to characterize them as distinct species.

Welch regards as belonging to this group the *Bacillus pyogenes fœtidus*, distinguished by the stinking odor of its cultures, and the *Bacillus lactis aërogenes*, which is characterized chiefly by its plumper form, its more energetic gas-production, its rapid coagulation in milk, and its denser growth in cultures.

Theobald Smith suggests that only those forms may be regarded as typical members of the group which grow on gelatin in the form of delicate bluish or more opaque whitish expansions with irregular margin, which are actively motile when examined in the hanging-drop from young surface colonies taken from gelatin plates, which coagulate milk within a few days; grow upon potato either as a rich pale or brownish-yellow deposit, or merely as a glistening, barely recognizable layer, and which give a dis-

tinct indol-reaction. Their behavior in the fermentation-tube[1] must conform to the following scheme:

[1] The fermentation-tube is a special form of culture-tube which may be obtained from dealers in bacteriological supplies. The closed branch of the tube should be completely filled with culture-fluid, but no more fluid should be placed in the tube than can be conveniently held by the open branch of the tube, so that if gas be formed in the closed branch the culture-fluid will not be forced out of the apparatus. The bubbles which collect at the top of the closed branch, after heating during sterilization, should be removed by an appropriate tilting of the tube. Theobald Smith, who was the first to demonstrate the great value of the fermentation-tube in bacteriology, thus describes the mode of its use:

"The tubes are kept, after inoculation, in the thermostat at 37° C. A mark made on the sides of the closed branch at the end of every twenty-four hours with a glass pencil furnishes an approximate record of the rate of gas-production. Unless this is done it is impossible to know precisely when the formation of gas is at an end, and also whether or not the volume of gas has been diminished by absorption. It is best to wait four or five days after the production has ceased before making a final examination. This is done by noting the condition of the growth, the reaction of the fluid in the bulb,* and the maximum quantity of gas produced. This is most easily done by laying directly on the tube a glass millimeter rule, and noting the tube length occupied by gas. The entire length of the closed branch is also noted, making due allowance for the upper convex extremity and the lower constriction. This mode of measurement is sufficient, since only comparative values are desired. For the same reason all barometric and thermometric corrections are omitted in these approximate estimations.

"The examination of the gas produced was limited to the determination of the quantity of carbon dioxid and of the explosive character of the gas remaining after the absorption of CO_2 by sodium hydrate. These facts are determined by the following simple manipulations:

"The bulb is completely filled with a 2 per cent. solution of NaHO, and closed tightly with the thumb. The fluid is shaken thoroughly with the gas, and allowed to flow back and forth from bulb to closed branch and the reverse several times, to insure intimate contact of the CO_2 with the alkali. Lastly, *before removing the thumb, all the gas is allowed to collect in the closed branch* so that none may escape when the thumb is removed. If CO_2 was present, a partial vacuum in the closed branch causes the fluid to rise suddenly when the thumb is removed. After allowing the layer of foam to subside somewhat, the glass scale is again applied to the closed branch, and the amount of CO_2 absorbed may thus be measured. In all cultures of this character thus far examined the gas remaining was explosive in character, and probably hydrogen. The explosive character of this residue is easily demonstrated as follows: The

*The reaction was noted by placing a drop of the fluid on delicate litmus-paper. The cultures were occasionally boiled to drive off any CO_2. In no case did the reaction with the litmus-paper change.

Variety α. One per cent. dextrose-bouillon (at 37° C.).
Total gas, approximately 1/2; H/CO_2 approximately 2/1; reaction strongly acid.
One per cent. lactose bouillon:
As in dextrose-bouillon (with slight variation).
One per cent. saccharose-bouillon:
Gas-production slower than in the preceding, lasting from seven to fourteen days. Total gas finally about 2/3; H/CO_2 nearly 3/2. The final reaction in the bulb may be slightly acid or alkaline, according to the rate of gas-production.

Variety β. The same in all respects excepting as to its behavior in saccharose-bouillon. Neither gas nor acids are formed in it.

Bacillus Dysenteriæ (Shiga).—This bacillus resembles the typhoid bacillus in morphology, but in general it is plumper and less frequently appears in filamentous forms. Involution forms quickly develop in glucose-agar cultures. It is decolorized by Gram's method, and does not form spores. It is not motile. The bacillus grows in bouillon and on agar and gelatin, both in plate and tube cultures, with appearances very similar to those of the typhoid bacillus. It does not produce gas in media containing glucose or other sugars.

Typical examples of the bacillus do not produce indol in peptone solution, but some strains have been found to do so.

Litmus Milk.—During the first two or three days the milk becomes a pink color, but later becomes blue. It is never coagulated.

Potato.—Growth, at first in the form of a moist, colorless, slimy, almost invisible layer, becoming, after two or three days in the incubator, of a yellowish to brownish tint with discoloration of the potato.

Mannite Litmus Agar.—This medium consists of ordinary nutrient agar-agar containing 1 per cent. of mannite and 1 per cent. of a 5 per cent. aqueous solution of litmus. The

cotton plug is replaced, and the gas in the closed branch allowed to flow into the bulb, and mix with the air there present. The plug is then removed, and a lighted match inserted into the mouth of the bulb. The intensity of the explosion varies with the quantity of air present in the bulb."

agar-agar should have been made up with meat infusion free from muscle-sugar (see p. 75). In stab cultures on this medium the typical bacillus decolorizes the agar in the depths, but near the surface the original blue color remains unchanged. Some strains of the bacillus, however, change the color of the upper layers to red, as do typhoid and colon bacilli. Thus two varieties of the bacillus may be distinguished with reference to their effect on this culture-medium. Both of these varieties have been isolated from the same dysenteric stools.

The bacillus exhibits the agglutination reaction with the serum of dysenteric cases and with the serum of animals immunized against the bacillus.

Pathogenesis.—The bacillus is pathogenic for the usual laboratory animals, especially for mice and guinea-pigs, which may die in from twenty-four to forty-eight hours after subcutaneous or intraperitoneal inoculation. At the autopsy there may be found local inflammation at the seat of inoculation, ecchymoses of the serous membranes, serous or sero-hemorrhagic exudate in the pleural or peritoneal cavities, enlargement of the spleen, and hyperemia or hemorrhage in the intestinal walls.

Occurrence.—In the stools, intestinal contents, and in the ulcerated mucous membrane of acute dysentery, whether sporadic or epidemic. It may be found in the mesenteric lymphatic glands, but is not found in the blood, in the spleen, or in other viscera.

Method of Isolation of the Bacillus.—A small portion of mucus is to be broken up in a tube of sterile bouillon. From the suspension of bacilli thus obtained agar-agar plate-cultures are prepared in dilutions depending upon the number of bacilli observed in smear preparations of the mucus. After twenty-four hours in the incubator transplants are made from *colonies resembling those of the typhoid bacillus* into 1 per cent. glucose bouillon contained in tubes arranged as suggested by Durham for the demonstration of gas-formation. These tubes are prepared as follows:

In an ordinary bacteriological test-tube, containing about one-third more than usual of 1 per cent. glucose bouillon, there is placed a small test-tube mouth downward. The larger tube is then plugged with cotton, and is sterilized by steam in the usual way. After the sterilization it will be found that the air has been driven out of the inverted inner tube and has been replaced by the bouillon. When cool, the tube is ready for inoculation. If the bouillon at the bottom of the larger tube be inoculated with a bacterium capable of fermenting glucose with the production of gas, this property of the bacterium will manifest itself by the collection of gas in the upper part of the small inverted inner tube.

As the dysentery bacillus does not produce gas from glucose, gas-formation in a tube is proof that the colony from which it was inoculated is not the dysentery bacillus. The bacteria in tubes in which no gas has developed are to be examined with reference to morphology and motility. If found to agree in morphology with the bacillus of dysentery and to be non-motile, then the agglutination test with dysentery serum may be applied to them immediately and they are to be transplanted on the various media to test their cultural peculiarities.

The advantages of this method over that of first transplanting the suspected colony into sugar-agar, as is usually done, are, first, that it enables the motility, agglutination reaction of the bacilli, and any gas-formation to be determined with the use of a single culture-tube; and, second, that it dispenses with the use of glucose-agar tubes, which are much more troublesome to prepare than are the bouillon tubes. These considerations are of considerable importance when a number of cases are being examined, for at least six colonies should be tested in each case as above described.

Bacillus Tuberculosis.—*Synonym:* Tubercle bacillus; Bacillus of Koch.

Blood-serum.—After three or four weeks in the incubator

the colonies appear as dry, cream-colored, granular, slightly elevated patches with irregular margins, 1 to 2 mm. in diameter. They may become confluent, to form a dense, dry, granular mass with irregular surface and of a creamy-white color. The growth is very friable, but coherent, and may be picked up in clumps on the platinum wire. The first generation from tissues is very slow in developing, but succeeding generations grow more rapidly, and may form a wrinkled, dry, cream-colored membranous layer on the surface of the medium.

Morphology.—Slender rods, usually shorter than when observed in sputum, and in fresh cultures staining homogeneously; in older cultures presenting a segmented or

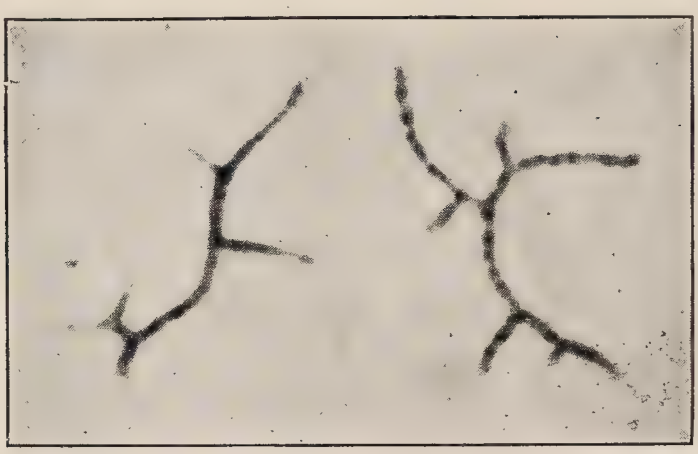


FIG. 62.—Branched tubercle bacilli from sputum; × 2000 (Wright and Brown).

irregularly stained appearance. They frequently occur in pairs of short rods and in closely adhering clumps and strands. When once stained with fuchsin or gentian-violet they are not decolorized by treatment with Gabbet's solution or with a 20 per cent. solution of any of the mineral acids, followed by alcohol. In the sputum of pulmonary tuberculosis the bacillus sometimes occurs in filaments which branch. On this account the organism is considered by many to belong to the group of the streptothrices.

Stained by Gram's method. Not motile. Does not form spores.

Glycerin Agar-agar Slant.—Growth similar to that on blood-serum, but not so vigorous. By continued inoculation of this medium through a number of generations,

however, the organism may eventually grow luxuriantly upon it.

Glycerin Bouillon.—Growth on the surface in the form of floating patches or as a membrane similar in appearance to the growth on blood-serum. The growth sinks to the bottom from time to time. The glycerin-bouillon culture is best contained in Erlenmeyer flasks, filled to such a depth as to give a wide surface to the fluid and thus permit the access of plenty of oxygen to the growth.

Potato.—The growth is not remarkable.

Agar-agar or bouillon not containing glycerin is not suitable for the cultivation of this bacillus.

Pathogenesis.—The inoculation of guinea-pigs or rabbits by any method is followed by the development of general miliary tuberculosis. Guinea-pigs are most susceptible. These animals usually survive about two or three months, with marked emaciation. The lesions in the spleen and liver in the guinea-pig are characterized by extensive areas of necrosis not confined to the tubercular tissue, large parts of these organs being transformed into a firm yellow, opaque, friable material.

Isolation of the Bacillus Tuberculosis from Tubercular Lesions.—The tubercular lesions in human tissues are not ordinarily favorable for the isolation of the bacillus, on account of the frequent presence of other bacteria in them and because of the small number of tubercle bacilli usually present in tissue otherwise suitable. The best method of procedure is to inoculate a guinea-pig subcutaneously in the abdominal wall with tubercular material, and after four to six weeks, when the inguinal lymphatic glands have become enlarged, to kill the animal and make cultures on suitable media from tuberculous lymphatic glands. The object of killing the animal, rather than allowing it to die spontaneously, is to secure fresh tissue and to avoid the chance of an invasion of the lesions by other bacteria.

For the cultivation of tubercle bacilli from tubercular lesions, M. Dorset highly recommends his egg-medium, which is prepared as follows:

Fresh eggs are broken under aseptic precautions into a wide-mouthed sterile flask, and the white and yolk mixed thoroughly. To every four eggs add 25 c.c. of sterile water. Any foam may be removed by straining the mixture through a sterile cloth. The mixture is then run into sterile tubes,—about 10 c.c. into each tube,—and slowly hardened in the form of "slants" in a blood-serum oven at a temperature of 73° to 76° C. This degree of temperature should be maintained for four or five hours on three successive days. On the first two days the temperature is maintained at 73° C., and on the third day at 76° C. Just before inoculating the medium three or four drops of sterile distilled water should be added to each tube to supply the moisture required for the satisfactory development of the tubercle bacillus.

After inoculation the tube should be placed in the incubator at 38° C. in an inclined position, so that the surface of the medium may keep moist. Colonies first become visible after seven or eight days in the incubator.

A number of tubes are to be inoculated, say three or four, from each of the two or three glands, a large quantity of material being spread upon the surface of each tube. Great care is to be exercised to avoid contamination with other bacteria in preparing these cultures. The culture-tubes used should contain freshly prepared moist medium, and immediately after inoculation should be sealed air-tight to prevent evaporation. This may conveniently be done by first cutting off the projecting portion of the cotton stopper and inserting a cork into the mouth of the tube in such a way as to push the cotton stopper before it.

In order to prevent the invasion of fungi from the cotton, the neck of the tube should be heated in the Bunsen flame until the cotton begins to brown before inserting the cork, which should also be charred in the Bunsen flame before insertion. The tubes may also be sealed with wax or paraffin or covered with small rubber caps.

Wolbach and Ernst have noted the following chief points

of difference between tubercle bacilli from human and from bovine lesions when cultivated on Dorset's egg medium. The human cultures as compared with the bovine cultures grow more profusely and the membranous layer that is formed is more nodular, drier looking, less translucent, more adherent to the medium, harder, and more difficult to break up with the platinum wire. The human bacilli generally show a slightly greater variation in length and thickness than do the bovine bacilli.

The Antiformin Method for Obtaining Pure Cultures of the Tubercle Bacillus.—Antiformin is the patented name for a solution consisting of equal parts of liquor sodæ chlorinatæ and a 15 per cent. solution of caustic soda. It quickly dissolves mucus and the cells and fibers of animal tissues, and also has the remarkable property of destroying all bacteria except tubercle bacilli and other acid-fast bacilli.

Lawrason Brown and Daniel Smith have used the following procedure with great success in cultivating tubercle bacilli from the sputum in a series of cases:

Equal parts of a 30 per cent. aqueous solution of antiformin and sputum are thoroughly mixed in a sterile centrifuge tube and allowed to stand at room-temperature for one hour. The tube is then centrifugalized, the supernatant fluid decanted, and the sediment mixed with sterilized distilled water. This is again centrifugalized, and the whole process is carried out three times. The sediment is then streaked over the surface of Dorset's egg medium and placed in the incubator.

Cultures from tubercular tissue may also be obtained by a similar procedure, the tissue being ground up in a mortar with a 15 or 20 per cent. solution of antiformin, or frozen sections made of it and placed in the same solution. When the tissue has been dissolved, which occurs in the course of a few minutes, the solution is to be centrifugalized, the sediment washed, and cultures made from it as above described for sputum.

Occurrence.—In tubercular lesions generally and in the

sputum of pulmonary phthisis, in the urine in many cases of genito-urinary tuberculosis, and in the feces in intestinal tuberculosis. The tuberculosis of cattle and of birds is due to different varieties of this bacillus.

Does not multiply outside of the body except in cultures.

May occur on the surface of objects contaminated with the excreta of tuberculous individuals or in the dust of places inhabited by such individuals.

Diagnosis.—For clinical purposes the tubercle bacillus may be identified in cover-glass preparations by means of the special methods of staining, which depend upon the fact that the bacillus tuberculosis, when once thoroughly stained with an aniline dye, does not give up its stain in the presence of acids, as nearly all other bacteria do. The bacillus tuberculosis may therefore be identified even among a mixture of other bacteria by this property, taken in connection with its morphology, in most of the routine work of the pathological laboratory. Practically, the only other bacilli with which it may be confounded are the bacillus of leprosy and the smegma bacillus, both of which, when stained, resist the decolorizing action of acid. It may be differentiated from the smegma bacillus by the fact that it is not decolorized by alcohol (95 per cent.) after the usual treatment with acid, while the smegma bacillus is decolorized under these circumstances.

As a rule, the differential test with alcohol need only be applied in the examination of urine and the material derived from about the external genitalia, especially in the case of females.

The differentiation from the bacillus of leprosy by certain quantitative differences in staining reactions has been attempted, but it is very unsatisfactory, and it is doubtful if there is as yet any reliable method of distinguishing between these two organisms, considered by themselves.

Examination of Sputum for Tubercle Bacilli.—The morning sputum should be taken for examination. Select one of the dense, grayish-white particles, and with

the aid of small-pointed forceps or the platinum wire rub it over the surface of a cover-glass, breaking it up as much as possible. The material should be spread in a very thin layer. The preparation is next to be "fixed" in the ordinary way described for cover-glass preparations (see p. 93), and is then to be treated as follows:

1. Stain in carbol-fuchsin solution, steaming for one minute over the Bunsen flame, with the staining solution thoroughly covering all the surface of the cover-glass. None of the surface of the cover-glass should be allowed to become dry by evaporation, as this causes a precipitate to form, but more of the staining fluid should be added from time to time to

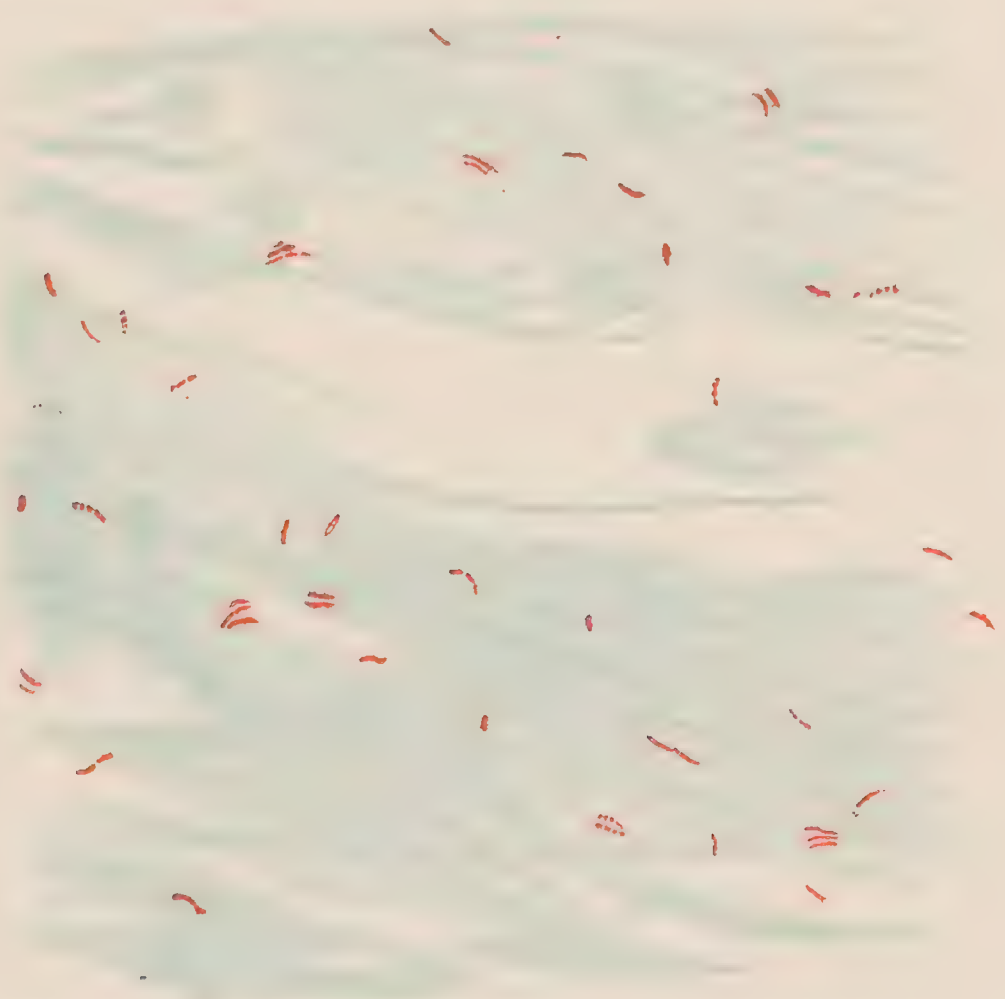


FIG. 63.—Tubercle bacilli in sputum (carbol-fuchsin and methylene-blue) (Vierordt).

keep it completely covered as evaporation occurs. The object of the heating is thoroughly to impregnate the bacilli with the dye.

2. Wash in water.

3. Decolorize in a 30 per cent. aqueous solution of concentrated nitric acid until the red color disappears. Do not allow the acid to act on the preparation longer than a few seconds. The solution should also be applied to the uncharged side of the cover-glass to remove any dried stain which may have collected thereon.

4. Wash thoroughly in water.

5. Wash in 95 per cent. alcohol for thirty seconds.

6. Wash in water.

7. Stain in Löffler's methylene-blue solution for thirty seconds.

8. Wash in water, dry, and mount in xylol balsam.

The tubercle bacilli are stained red and the nuclei of cells and other bacteria are stained blue.

Antiformin Method.—The finding of tubercle bacilli in sputum is greatly facilitated by making cover-glass preparations, as above described, from the sediment obtained by the antiformin method described on page 183.

In order to destroy any extraneous tubercle bacilli or other acid-fast bacilli which may be in the centrifuge tube, just before use the interior of the tube is to be thoroughly exposed to the action of concentrated sulphuric acid saturated with potassium bichromate, after which the tube is to be thoroughly washed with distilled water. Sputum cups, or other receptacles of crockery or of glass used to collect the sputum, should be treated in the same way.

It is of the greatest importance to be sure that the distilled water used does not contain acid-fast bacilli which sometimes develop in it.

As antiformin does not kill tubercle bacilli, the centrifuge tube should be sterilized after use.

In a very few cases of gangrene of the lung bacilli like smegma bacilli have been found in the sputum. These may be mistaken for tubercle bacilli (*vide ante*).

Tubercle Bacilli in Urine.—The sediment of the urine should be examined. This may be rapidly thrown down by the centrifuge. With the sediment, cover-glass preparations are to be made and stained as described for sputum. Especial care should be taken to wash thoroughly in alcohol after the decolorization with acid, in order to decolorize any smegma

bacilli that may be present. (See remarks on Diagnosis, p. 184.) Because smegma bacilli may be mistaken for tubercle bacilli and because the tubercle bacilli may be so few as to escape observation, the inoculation of a guinea-pig with the sediment is the better test for the presence of tubercle bacilli in the urine. For this purpose the urine should be collected in sterilized vessels and immediately centrifugalized in sterilized tubes. The sediment is then to be injected subcutaneously into a guinea-pig with a sterilized syringe. (See p. 118.)

Tubercle Bacilli in Tissues, Pus, and Feces.—The bacilli may be demonstrated in the following ways:

1. By the staining of the bacilli in sections of tissue by the special methods described on pages 434 to 436. Frozen sections prepared by the method described on page 271 may be employed.

2. By making cover-glass preparations and staining as described for sputum. These preparations may be made directly from the material; but if the bacilli are few, as is usually the case, they should be made from the sediment obtained by the antiformin method described on page 183. This method is also applicable to fixed and hardened tissue, even if it has been imbedded in paraffin. The paraffin should be thoroughly removed from the sections by means of xylol, followed by absolute alcohol, before placing them in the solution of antiformin.

The precautions against error from the presence of extraneous tubercle bacilli, or other acid-fast bacilli, are to be taken which are described in connection with the application of this method to the examination of the sputum.

3. By the inoculation of guinea-pigs with the material or sediment obtained by the antiformin method. The inoculation is best made subcutaneously in the abdominal wall, either with a hypodermic syringe, if the material be fluid, or if it is in the form of tissue, by inserting a small piece beneath the skin. Material obtained on a swab may also be used for inoculation by introducing the infected swab beneath

the skin and moving it back and forth a few times. If tubercle bacilli are present in the material, the animal will show enlargement of the inguinal lymphatic glands in about three weeks, and will usually die of miliary tuberculosis in the course of six to ten weeks. If necessary, the glands in the inguinal region may be examined histologically after three weeks for the presence of tubercular lesions, or examined by cover-glass preparations for tubercle bacilli.

Bacillus of Leprosy.—This bacillus resembles closely the tubercle bacillus in morphology and staining reactions. It is somewhat less resistant than that bacillus to decolorization by acids.

Cultures.—There is no satisfactory evidence that this organism has ever been obtained in pure culture.

Pathogenesis.—The Japanese waltzing mouse is the only animal known to be very susceptible to inoculation with this bacillus. The inoculation of this animal is followed by extensive and widespread nodular lesions containing large numbers of bacilli, and very similar in histological character to those of the disease in man.

Occurrence.—The bacillus often grows in enormous numbers in the lesions, and chiefly in the cytoplasm of mononuclear cells, where they often lie parallel to one another in bundles. They may also be found in nerves and in nerve-cells. In lesions of the nasal mucosa they may be demonstrated in the secretion.

Spirillum of Asiatic Cholera (Comma Bacillus).—*Morphology* (Figs. 64, 65).—In fresh cultures the organism appears usually as a slightly curved rod somewhat shorter than the tubercle bacillus, but much thicker. The curving of the rod varies, being very marked in some individuals and absent in others. Sometimes two rods are joined end to end with their convexity pointing in opposite directions, or moderately long, undulating threads may be found. It seems probable that the curved rods represent the segments of a spirillum, and hence the name of the organism.

In cultures some days old degenerated and atypical forms

are found (involution forms). The organism is motile, and a single flagellum is attached to the end of the rod.

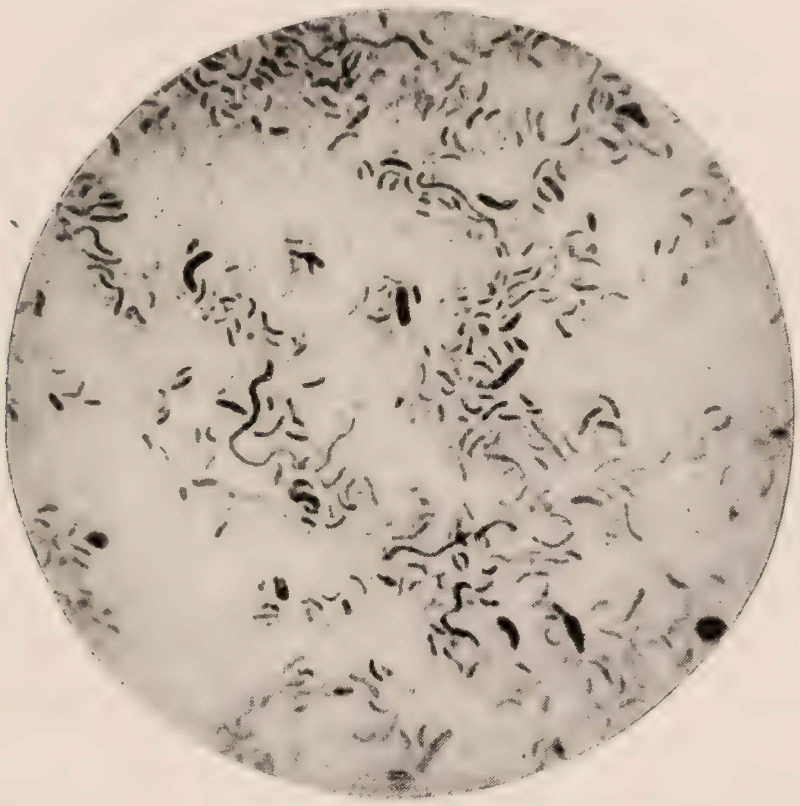


FIG. 64.—Spirillum of Asiatic cholera, from a bouillon culture three weeks old, showing long and degenerate forms; × 1000 (Fränkel and Pfeiffer).

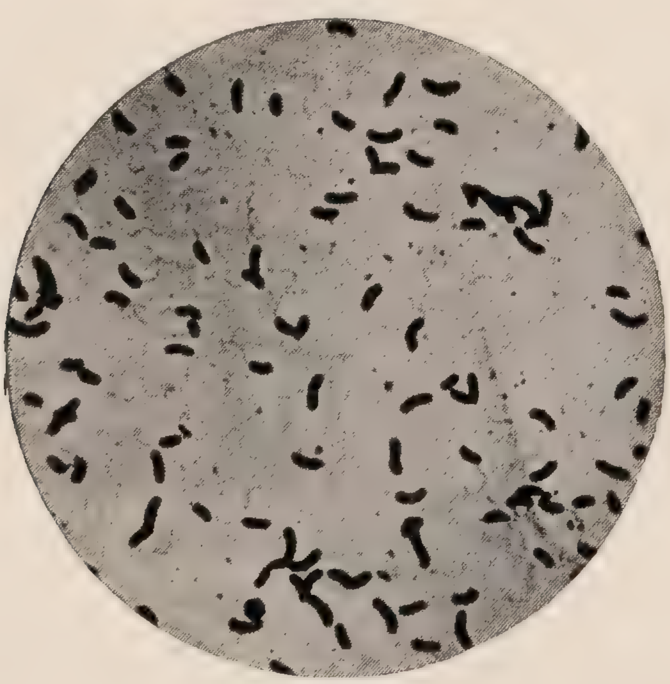


FIG. 65.—Spirillum of Asiatic cholera, showing the flagella; × 1000 (Günther).

It is not stained by Gram's method.

Colonies on Gelatin Plates (Fig. 66).—After twenty-four to forty-eight hours at a temperature of 20° to 22° C. the

largest colonies will appear as masses of indefinite granular material lying in circular areas of liquefied gelatin in which granular shreds are scattered. Within the next twenty-four hours the areas of liquefaction increase, and the colonies ap-

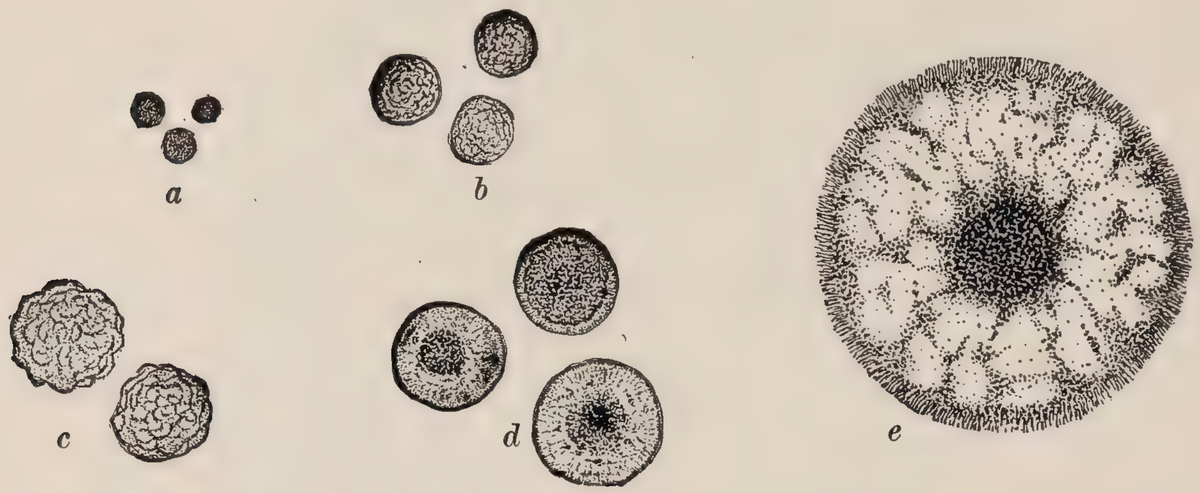


FIG. 66.—Developmental stages of colonies of the spirillum of Asiatic cholera at 20° to 22° C. on gelatin; × about 75 diameters (Abbott): *a*, after sixteen to eighteen hours; *b*, after twenty-four to twenty-six hours; *c*, after thirty-eight to forty hours; *d*, after forty-eight to fifty hours; *e*, after sixty-four to seventy hours.

pear under the low power "as a dense granular mass surrounded by an area of liquefaction through which can be seen granular prolongations of the colony, usually extending irregularly between the periphery and the central mass" (Abbott), while the margin of the liquefied area is marked by delicate radiating filaments closely packed together.

The colonies on agar-agar plates are not characteristic. Growth is rapid.

Gelatin Stab.—Growth all along the line of inoculation with liquefaction at the surface in funnel form after forty-eight hours. The liquefaction proceeds in such a manner that the liquefied area has a smaller diameter at the surface than immediately beneath, and, owing to the fact that the liquefied gelatin does not fill the cavity, a space is left between the surface of the medium and the surface of the liquefied gelatin so that the appearance of an air-bubble is produced. Along the deeper portions of the line of inoculation the liquefaction is slow.

Bouillon.—Diffusely clouded. A thin pellicle forms on the surface after a time.

Litmus-milk.—Turned red and coagulated.

Indol-production.—In cultures in Dunham's pepton solution or in the pepton solution of Koch (2 per cent. pepton and 1 per cent. sodium chlorid) a rose-color is produced by the addition of sulphuric acid alone. (Concentrated c. p. acid is to be employed, as in the test for indol-production by the bacillus coli communis.) The production of the rose-color without the addition of the sodium nitrite shows that nitrites as well as indol are formed by the growth of the organism in the pepton solution. The reaction can be obtained in cultures which have been but eight hours in the incubator.

Potato.—Thin, dry, grayish-white growth which does not spread over the surface.

Pathogenesis.—The pathogenic effects of the cholera spirillum are best shown by the inoculation of guinea-pigs. There are two methods of inoculation, as follows:

1. *The Method of Pfeiffer.*—Scrape from the surface of a fresh agar-agar culture as much of the growth as will adhere to a platinum wire bent into the form of a small loop. Suspend this amount of material in 1 c.c. of bouillon, and inject the suspension into the peritoneal cavity of a guinea-pig by means of a hypodermic syringe. With virulent cultures this inoculation soon produces a fall in the temperature of the animal, which continues and becomes more marked, death occurring in from twelve to twenty-four hours. At the autopsy of the animal a clear fluid will be found in the peritoneal cavity and in the thorax.

2. *The Method of Koch.*—This depends upon the fact that the animal may be infected through the alimentary canal, provided the acidity of the gastric juice be neutralized, this acidity being destructive to the cholera spirillum.

A soft catheter is passed into the stomach of the animal through the mouth, and through this 5 c.c. of a 5 per cent. solution of sodium carbonate is injected. After ten or fifteen minutes 10 c.c. of a bouillon culture of the organism are injected through the catheter, and immediately afterward the animal receives subcutaneously 1 c.c. of the tincture of opium for every 200 grams of its body-weight. The object of this opium administration is to stop peristalsis, so that the

organisms may be longer in contact with a given area of the mucous membrane of the intestine. The result of the inoculation first appears after about twenty-four hours. The animal then has no appetite and is listless. Later, paralysis of the hinder extremities appears, respiration is prolonged and weak, the heart-beats become feeble, and the body-temperature may become subnormal. Death usually occurs after the animal has been a few hours in this condition.

At the autopsy the small intestine will be found to be injected and containing a flocculent colorless fluid in which comma bacilli are present in great numbers.

Agglutination Reaction.—Cholera spirilla cease their motion and aggregate together in clumps, when a small quantity of the blood-serum of an animal immunized against them is added to a suspension of them in salt solution. This phenomenon is called the agglutination reaction.

An agglutinating cholera serum may be produced by injecting into the ear-vein of a healthy rabbit increasing quantities of the growth from eighteen-hour agar cultures of known cholera spirilla in suspension in normal salt solution; these suspensions must have been heated for one hour at 60° C. The quantities and intervals are: first day, 1 loopful of the growth; seventh day, 3 loopfuls; fourteenth day, 5 loopfuls; twenty-first day, about 8 loopfuls. The fourth injection may be made into the peritoneal cavity and the rabbit is ready to be bled on the twenty-eighth day. This procedure should give serum which will agglutinate cholera spirilla in a dilution of 1 : 4000. The serum should be collected under precautions to prevent contamination by bacteria, and should be kept in small sealed tubes on the ice.[1]

Pfeiffer's Reaction.—One loopful of an eighteen-hour culture of the cholera spirillum is suspended in 1 c.c. of a dilution of agglutinating cholera serum in normal salt solution, which is somewhat less dilute than the maximum dilution

[1] These directions are essentially those given by A. J. McLaughlin. Reprint from *Public Health Reports*, No. 53. Public Health and Marine Hospital Service of the United States.

necessary to agglutinate the cholera spirillum. This mixture is injected into the peritoneal cavity of a guinea-pig of about 200 gms. weight. After about twenty-five minutes some of it is withdrawn and examined microscopically, when the spirilla will be found to have lost their motility and to have become swollen and of degenerate appearance. Ultimately they disintegrate and disappear. Other spirilla do not undergo this change, and the reaction is specific.

Occurrence.—In the alvine dejections and in the intestinal contents of cholera patients (Fig. 67). It apparently only

FIG. 67.—Cover-glass preparation of a mucous floccule in Asiatic cholera; × 650 (Vierordt).

rarely invades the circulating blood. Its presence in the vomitus may sometimes be shown. It has been found in the water-supplies during epidemics.

The cholera spirillum is the representative of a large group of spirilla, many of which may be found in river waters. According to Abbott and Bergey, the only trustworthy method of distinguishing some of these from the true cholera spirillum is their failure to manifest a "clump reaction" with the serum of an animal immunized to infection with the true cholera spirillum.

Bacteriological Diagnosis.[1]—Cultures should be made from the feces, or contents of the lower end of the ileum, in a special fluid medium and on agar Petri plates. Mucus flakes, if possible, should be taken for inoculation. The special fluid medium favors the growth of spirilla and is prepared as follows:

Peptone (Chapoteau or Witte)	10.0
Salt	10.0
Potassium nitrate	.1
Sodium carbonate	.2
Distilled water	1000.0

The agar plates are made up with 15 c.c. each of 3 per cent. agar, which has been made alkaline by the addition of 3 c.c. of a 10 per cent. solution of caustic soda to each 100 c.c. of the medium after it has been made neutral to litmus. The plates are inoculated in sets of three after the agar has solidified by rubbing one loopful over the surface of the agar in one plate with a platinum loop or a bent glass rod, and then streaking the surfaces of the other plates successively with the same loop or rod. The surfaces of the solidified agar must be dried before inoculation by placing the plates for five minutes in a warming oven at 60° C., or in the incubator at 37° C., for one hour, with the covers removed and the agar surface downward. The tubes of the fluid medium should be inoculated with one loopful of the material and the flasks with 1 c.c.

After inoculation all cultures are placed in the incubator at 37° C.

The uppermost layers of the fluid cultures should be examined microscopically after three, six, twelve, and twenty-four hours without disturbing the fluid more than is necessary. If spirilla are found agreeing in morphology, motility, and staining reactions with the cholera spirillum, agar plates are to be inoculated from this uppermost layer in the same manner as from the original material and incubated at 37° C.

The colonies on the agar plates develop within eighteen hours, and appear as pale, semitransparent discs, which show by transmitted light an opalescent or iridescent quality. Suspicious colonies are to be tested as follows: On a clean glass slide are placed at three separate points single drops of a 1 : 200 dilution in physiological salt solution of an agglutinating cholera serum, such as is described on page 192. These drops are numbered on the slide 1, 2, and 3. With them are then mixed portions of suspicious colonies, correspondingly numbered on the slide, by means of a straight platinum wire. If the diffuse cloudiness of the drop of fluid changes within a few minutes to a clear fluid with flocculi in suspension, and the macroscopical and microscopical appearances

[1] A. J. McLaughlin, *loc. cit.*

of agglutination are produced, the colony is probably that of the cholera spirillum. It may be necessary to test in this way numerous colonies. From colonies thus giving a positive agglutination reaction agar slants are inoculated and incubated for eighteen hours, when emulsions of the spirilla for more delicate agglutination tests are prepared by pouring into each tube 5 to 8 c.cm. of sterile physiological salt solution and shaking the tubes. Suspicious colonies not showing agglutination reactions should also be planted on agar slants and the growth tested again, because freshly isolated cholera spirilla do not always respond to the test.

The more delicate agglutination tests are carried out as follows: In each of a number of small test-tubes of 2 c.cm. capacity is placed ½ c.c. of dilutions in salt solution of agglutinating serum varying from 1 : 10 to 1 : 4000, or up to the limit of the agglutinating power of the serum. To each tube is then added ½ c.c. of the emulsion of the suspected spirilla. These manipulations are carried out with a pipette, to which is attached a rubber bulb for suction and expulsion. The highest dilutions at which agglutination appears in the tubes is noted after they have been in the incubator at 37° C. for one hour, and again after an additional two hours at room temperature. If the spirilla are true cholera spirilla, they will be agglutinated at or near the maximum dilution at which the specific serum agglutinates the true cholera microorganism.

Pfeiffer's reaction may also be employed as a confirmatory test. Of course the spirilla should also be shown to manifest the other characteristics described above before a positive diagnosis is made.

Dieudonné's Blood-agar Medium.—This has an inhibiting effect on the growth of other micro-organisms than spirilla, and may be employed in the same manner as the agar medium described above. It is prepared as follows:

Defibrinated ox blood	30
Normal solution of caustic potash	30
Nutrient agar (3 per cent.)	140

Add the caustic potash solution to the ox blood and add the melted agar. Sterilize for one hour at 100° C., and use about 15 to 20 c.c. for each plate.

Bacillus of Anthrax. — *Blood-serum.* — Irregularly rounded colonies, several mm. in diameter after twenty-four hours in the incubator. The colonies are grayish, finely granular, and have the appearance of being made up of a dense network of delicate fibrillæ. The blood-serum is slowly liquefied.

Morphology.—The organism grows in long segmented threads, the segments varying in length, but usually being two or three times as long as broad and having square or slightly concave ends. These segments represent the bacillus, which is among the largest of the bacteria (Fig. 68).

Pathogenesis.—Mice, guinea-pigs, and rabbits inoculated subcutaneously die with a general invasion of the blood by the organism. Mice are most susceptible to the infection, dying in about twenty-four hours, while guinea-pigs and rabbits survive longer.

FIG. 68.—Bacillus of anthrax: portion of a colony three days old upon a gelatin plate; × 1000 (Fränkel and Pfeiffer).

In all these animals the most striking lesion is a large soft spleen, and in the guinea-pig also an extensive inflammatory edema of the subcutaneous tissues. On microscopic examination the bacilli will be found in the organs and blood of the heart. If the animal has been dead some time,* the number of bacilli present in these situations will be very great, owing to the post-mortem growth. It is characteristic of the bacillus of anthrax in cover-slip preparations from infected tissues that it should have a narrow capsule (Fig. 69).

and show square or slightly concave ends. The capsule is not present in cultures.

Stained by Gram's method. Not motile.

Forms oval spores in the middle of the short segments or rods. The spores may be seen in blood-serum cultures after forty-eight hours in the incubator (Fig. 70).

Gelatin Stab.—Growth along the line of stab, with radiating filaments extending laterally into the gelatin, which is slowly liquefied in funnel form (Fig. 71).

Bouillon.—Growth in the form of cotton-like flakes and filamentous masses. No clouding of the medium.

Agar-agar.—Matted network of translucent filaments.

FIG. 69.—Bacillus of anthrax from spleen of a mouse (L. Frothingham).

Under a lower magnifying power the growth is seen to be made up of twisted and contorted masses of filaments, giving the appearance of curled hair (Fig. 73).

Potato.—Grayish-white, rather thick, dry layer, having the appearance of frosted glass.

Occurrence.—In malignant pustules, wool-sorter's disease, and intestinal anthrax. Found in the blood of animals dead

of anthrax. In man the infection is usually localized at first at the point of inoculation, either on the skin or on the

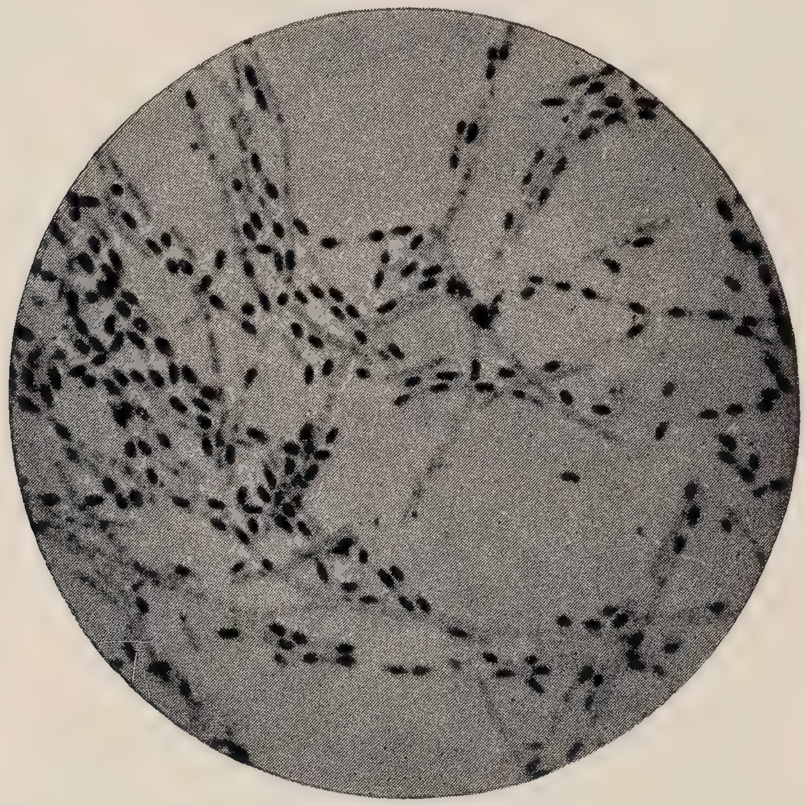


FIG. 70.—Bacillus of anthrax, stained to show the spores; × 1000 (Fränkel and Pfeiffer).

mucous membrane of the air-passages or intestinal tract. Later, a general invasion of the blood may occur and a fatal

FIG. 71.—Bacillus of anthrax: gelatin stab-culture seven days old (Günther).

septicemia result. The organism or its spores may be present in wool or hides, and infection may take place from these.

Diagnosis.—The bacilli may be found by the cover-glass

examination of the contents of the small blebs and vesicles. The bacillus of anthrax may be identified by its morphology (see p. 196), its special characteristics being its large size and its square or concave extremities.

The inoculation of a mouse at the root of the tail with some of the material from the pustule, and the production of the characteristic fatal septicemia, will render the identification certain.

FIG. 72.—Bacillus of anthrax; cover-slip preparation from vesicle. Stained by W. H. Smith's method; × 1800 (W. H. Smith; photo. by L. S. Brown).

FIG. 73.—Colony of bacillus of anthrax, slightly magnified (Flügge).

Bacillus Pyocyaneus (Bacillus of Green Pus).—Colonies on *blood-serum* grow rapidly, are not especially characteristic in form, and liquefy the medium, imparting to it a dark greenish color.

Morphology.—Small bacilli with rounded ends (Fig. 74). Decolorized by Gram's method (Welch). Motile, and is provided with a flagellum at one end. Does not form spores.

Gelatin Stab.—Liquefaction in funnel form, with green fluorescence of the upper portions of the medium. The liquefied gelatin is densely clouded, and there may be a viscid pellicle on the surface.

Agar-agar Stab.—A green fluorescence in the upper layers of the medium, which later becomes a dark blue-green.

Potato.—Slightly elevated, brownish, viscid layer. The potato in some cases assumes a green color, in others a brown color. In some cultures the potato when touched with the platinum wire takes on a green color at the point touched. This is the so-called "chameleon phenomenon," and it is best observed in cultures several days old.

Bouillon.—The growth is in the form of flocculi and a delicate surface pellicle. The fluid acquires a green color.

Litmus-milk.—Acid reaction with coagulation.

Dunham's Pepton Solution.—Indol is produced.

Colonies on Gelatin Plates (Fig. 75).—Development is rapid. Young colonies are provided with a fringe of delicate filaments about their periphery. As growth progresses and liquefaction becomes more advanced, the central mass of the

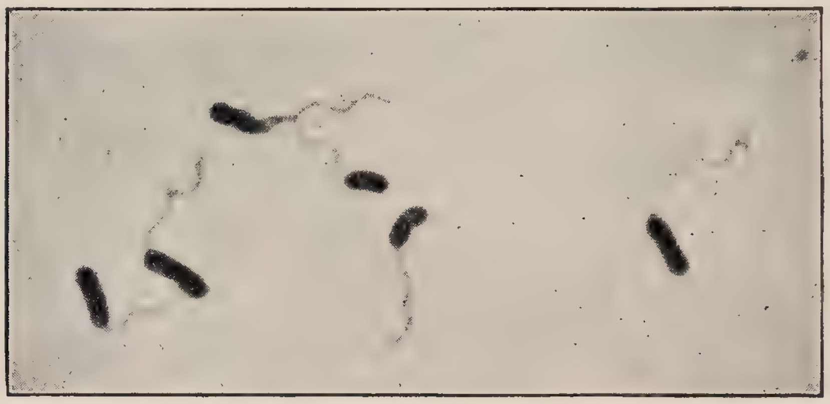


FIG. 74.—Bacillus pyocyaneus showing flagella, from a preparation stained by Dr. Hugh Williams; × 2000 (Wright and Brown).

colony sinks into the liquefied depression, while at the same time there is an extension of the colony laterally. . . . At this stage the colony, when slightly magnified, may present various appearances, the most common being that shown in Fig. 75. The gelatin between the growing colonies takes on a bright yellowish-green color, but, as growth is comparatively rapid, it is quickly entirely liquefied, and one often sees the colonies floating about in the pale-green fluid.

Pathogenesis.—Subcutaneous inoculation of guinea-pigs and rabbits with 1 c.c. of a virulent bouillon culture may produce purulent infiltration and inflammatory edema of the tissue about the point of inoculation, and death may follow in eighteen to thirty-six hours. Intraperitoneal inoculation may result in a sero-fibrinous or purulent peritonitis with

fatal result. In fatal inoculations the bacillus is found in the various viscera, but not in great numbers. Animals inoculated with small amounts may survive with merely local lesions, and an immunity may be produced.

Several varieties of this bacillus have been described, but their differences do not seem to be of sufficient importance to justify their separation into distinct species.

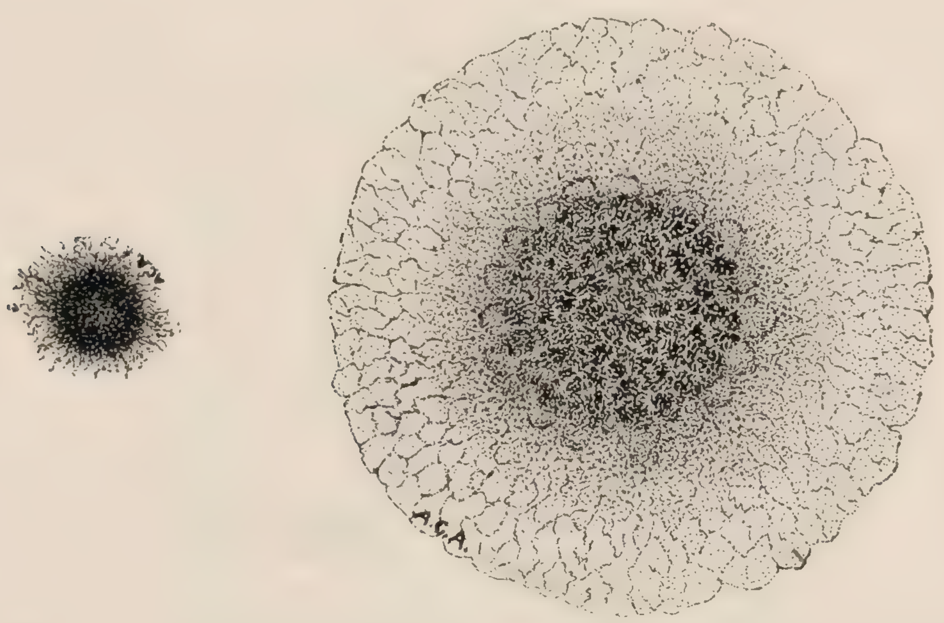


FIG. 75.—Bacillus pyocyaneus: colonies upon gelatin (Abbott).

Occurrence.—" Is widely distributed, occurring often on the human skin, in the feces, and outside of the body. In wounds, stains the dressings bluish-green and produces a somewhat characteristic offensive odor.

" Increases suppuration of wounds, usually with little constitutional disturbance. Is found not infrequently in perforative peritonitis and appendicitis, sometimes in phlegmons, otitis media, broncho-pneumonia, and inflammation of serous membranes, associated usually with other bacteria.

" It was found by H. C. Ernst in tuberculous pericarditis. Often found in diarrheal and dysenteric discharges. May cause general infection in human beings. With or without general infection it may cause hemorrhagic and necrotic enteritis, a form of pyocyaneous infection in human beings which we have repeatedly observed at autopsy. Instances of invasion of the body from wounds by the bacillus pyocyaneus have not been observed" (Welch).

Bacillus of Bubonic Plague.—*Morphology.*—In the

tissues the organism occurs as a medium-sized short bacillus with rounded ends. In cultures its size and length vary and its median portion may be swollen so that an ovoid form is produced; it may grow in pairs and in chains, and it may occur as long, thread-like forms. Involution forms of elliptical or round shape, and often of large size, sometimes resembling yeast-cells, are frequent in old cultures or in cultures on special media. These involution forms are easily produced by cultivation on agar-agar containing 2½ to 3½ per cent. of sodium chlorid.

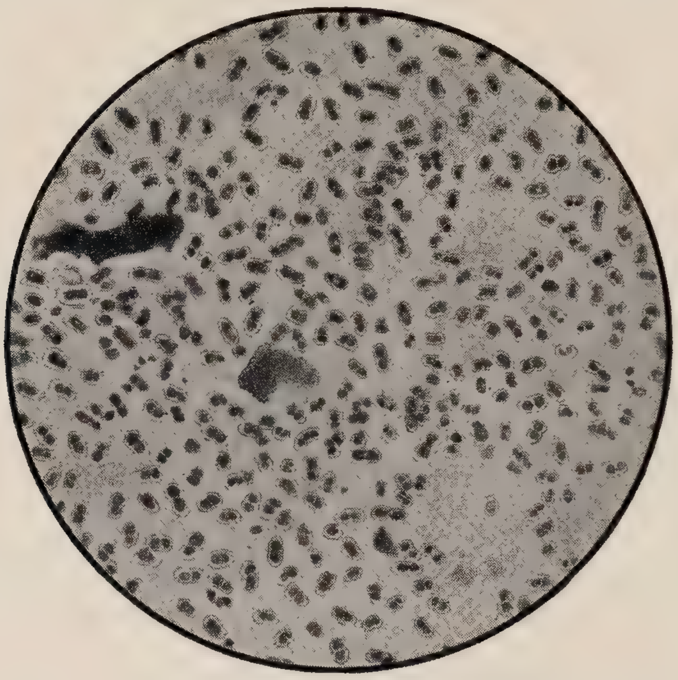


FIG. 76.—Bacillus of bubonic plague (Yersin).

Staining.—The organism stains with the usual aniline dyes, and is decolorized by Gram's method of staining. In the tissues it stains more deeply at its extremities than at its central portions, and it sometimes appears to possess a capsule. The polar staining may sometimes be brought out in cultures by weak staining solutions or by decolorization by alcohol. It is not motile, and it does not form spores.

Gelatin Plates.—The colonies on the surface appear after twenty-four to forty-eight hours at 22° C. They are flat, round, and white or yellowish white in color. Under a low magnifying power the central portion of the colony is granular, while the marginal portion is clear. The colonies do not spread over the surface of the medium.

Gelatin Stab.—Growth all along the line of inoculation

with the formation of a layer of growth at the surface of a whitish color. There is no liquefaction of the gelatin.

Gelatin Slants.—A whitish or slightly yellowish layer presenting nothing characteristic.

Agar-agar Plates.—The colonies on the surface appear first as dew-drops, and have already attained their maximum development after twenty-four to forty-eight hours in the

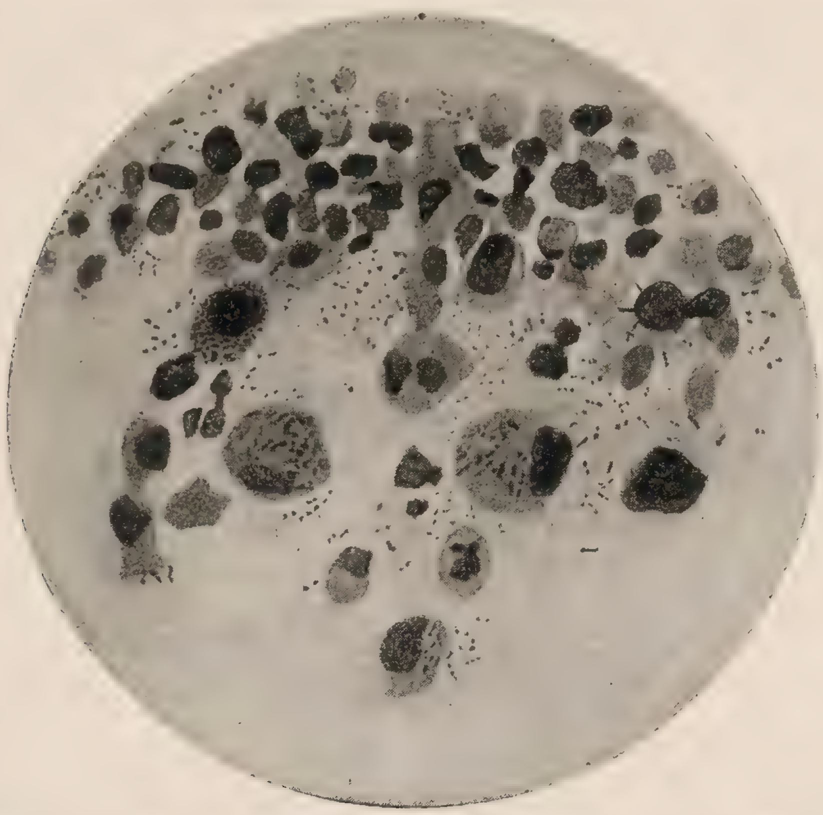


FIG. 77.—Bacilli of plague and phagocytes, from human lymphatic gland; × 800 (Aoyama).

incubator. They will then grow white in color, and present an opalescent or iridescent margin. Under the microscope they are distinctly granular. Considerable difference in size may be observed among the colonies. The larger colonies are said to be less virulent for animals than the smaller colonies, and it is claimed that these larger colonies when transplanted give rise to large colonies again.

Agar-agar Slant.—The colonies tend to become conflu ent, and the growth is somewhat viscid.

Bouillon.—The fluid usually remains clear, and the growth appears in the form of a granular or flocculent sediment which may here and there adhere to the wall of the tube. In bouillon cultures richly inoculated and retained in a perfectly and undisturbed position at room-temperature for

some days a characteristic appearance is produced. In twenty-four to forty-eight hours islands of growth appear underneath the surface in the form of flakes. In the next twenty-four to forty-eight hours there grow down into the fluid from the flakes long, stalactite-like masses, the liquid remaining clear. In four to six days the islands of growth have become more compact and solidified. If the flask be now slightly disturbed, the islands fall to the bottom, bringing with them the stalactite-like growths. The latter are very fragile. In addition to these appearances there is a deposit of growth on the wall of the flask and at the bottom, as well as a ring of growth on the margin of the surface of the liquid.

Milk.—Growth without coagulation.

No production of indol.

In neutral litmus bouillon the blue color is changed to red.

There is no odor, and no pigment production.

The organism is aërobic.

It remains alive in cultures for five to six weeks at least.

Growth occurs at all temperatures from 4° C. to 37° C. The best temperature for growth is 30° to 32° C.

Pathogenesis.—The organism is pathogenic for a great variety of animals, including mice, rats, guinea-pigs, and rabbits. In these animals death generally follows in from two to six days after subcutaneous inoculation. The lesions produced are hemorrhagic edema at the seat of inoculation, enlargement of the lymphatic glands with more or less hemorrhage, enlargement of the spleen and its follicles. The bacilli are present in large numbers in the enlarged lymphatic glands and in the internal organs; they are less numerous in the blood. Rats and certain other animals may be infected by feeding. Pigeons, chickens, and cattle are immune.

Occurrence.—The bacillus is found in large numbers in the buboes, pustules, pulmonary lesions, and other localized lesions of the bubonic plague. It also may be found in larger or smaller numbers in the blood and internal organs generally, and it may be present in the sputum, bile, and

alvine discharges. The pus of the buboes which break spontaneously may be sterile. The organism may be demonstrated in the circulating blood of cases of plague.

Bacteriological Diagnosis.—In cases of suspected plague the bacillus is to be sought for in the blood and in the buboes. In cases of pneumonia the sputum especially is to be examined. In the examination cultures as well as cover-glass preparations are to be used.

Perhaps the most certain method of identification of the bacillus is the inoculation of the mucous membrane of the nose of the rat. The simple rubbing of a portion of the culture upon the mucous membrane appears to be sufficient to produce a fatal result in the rat if the culture is that of the genuine bacillus. As a culture-medium agar-agar or blood-serum is to be used in cases where there is no mixed infection. If there is mixed infection of the material to be examined, gelatin surface-cultures are to be made.

The inoculation of animals for diagnostic purposes should be made with the greatest precaution to prevent the spread of the disease.

Bacillus of Influenza.—*Morphology.*—Very small bacilli, with rounded ends and of variable length, sometimes growing into long forms, more or less bent or curved (see Fig. 78).

Stains more deeply at the ends than in the middle, and in the long forms shows irregularity of staining. The faintly stained areas are very sharply defined, as in the case of the typhoid bacillus.

In cover-glass preparations from bronchial secretions (see Fig. 79), the bacillus appears smaller and less plump than it does in preparations from cultures. It also does not show irregularities in staining.

Cultivation.—Does not grow in the ordinary culture-media, but may be cultivated on agar-agar "slants," the surfaces of which have been smeared with a few drops of sterile blood. The blood of man, rabbits, guinea-pigs, pigeons, or frogs will serve for this purpose, the best growth being obtained with pigeon's blood. The blood may be obtained from a needle-prick, and spread over the surface

of the agar-agar by means of the platinum loop. The skin should be previously thoroughly washed with alcohol and

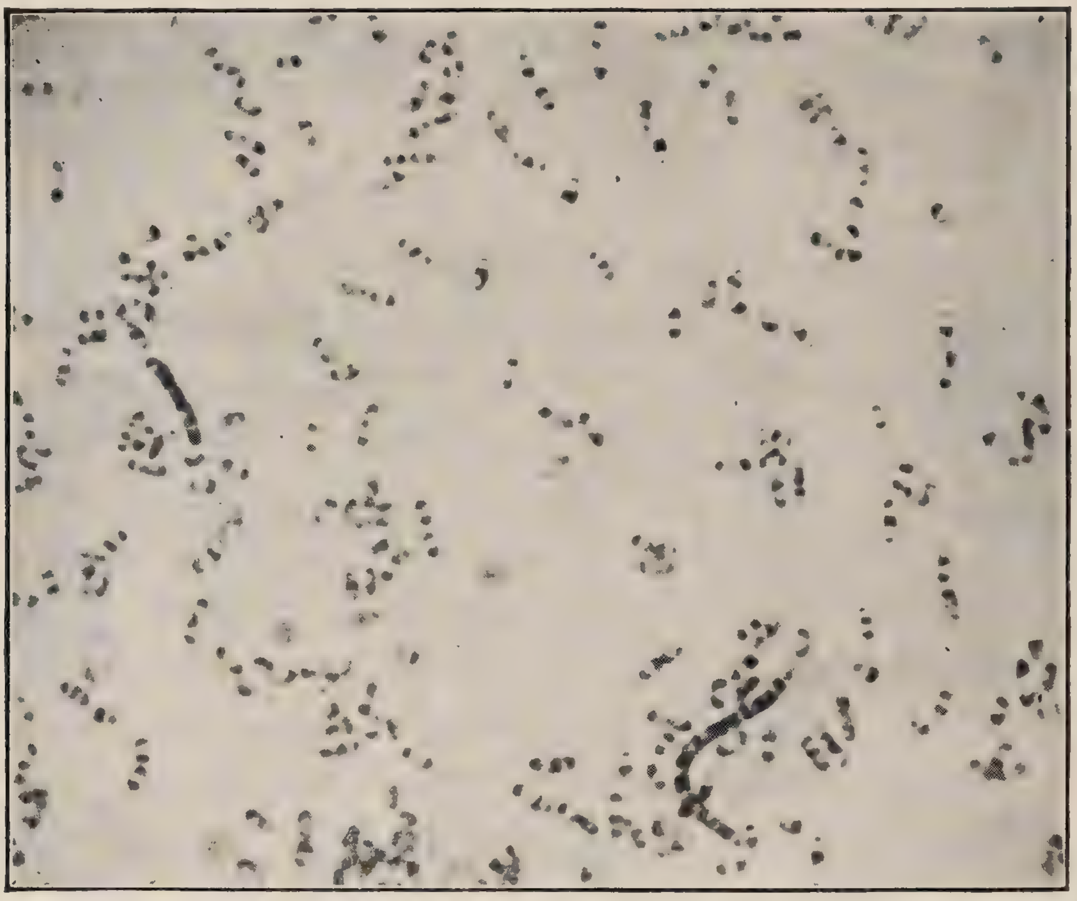


FIG. 78.—Influenza bacilli from a culture on blood-agar; × 2000 (Wright and Brown).

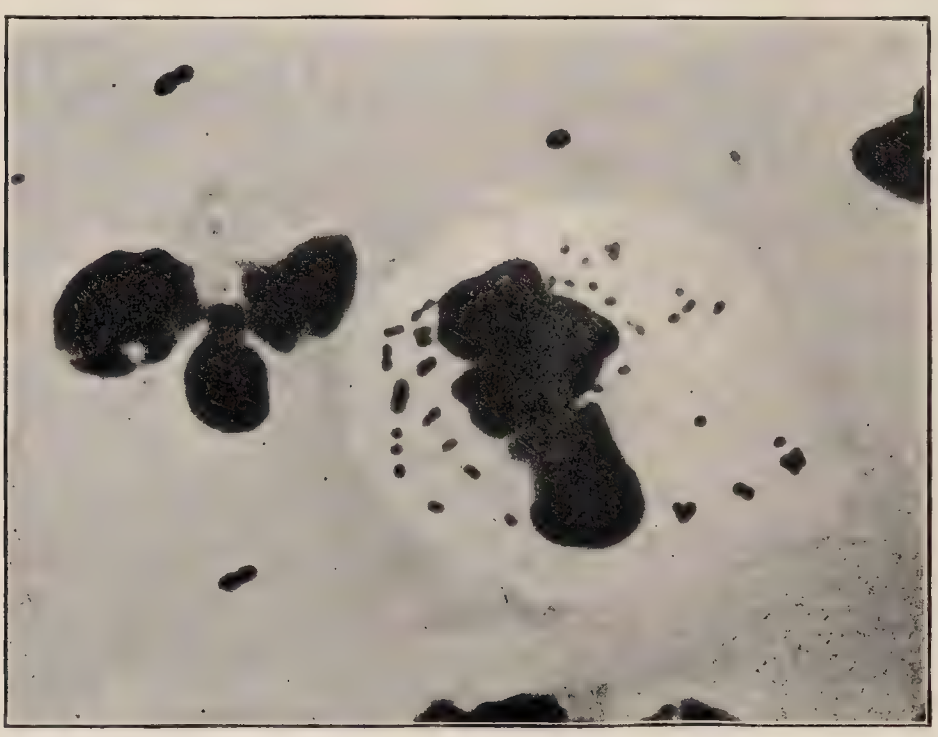


FIG. 79.—Bacilli of influenza in a leucocyte in a cover-glass preparation from sputum. A pneumococcus also in the same leucocyte and other pneumococci free. The small size of the bacillus of influenza will be apparent by comparison with the pneumococci; × 2000 (Wright and Brown).

ether, and the first drops of blood should not be used. Human blood is best obtained from the lobe of the ear

or from the finger. Tubes thus prepared are only rarely contaminated.

Colonies.—After twenty-four hours in the incubator the colonies appear as minute colorless, glassy, transparent points resembling small drops of dew. They never attain any size, and do not become confluent. They are barely visible to the unpractised eye, and require a low magnifying power to be seen clearly. Under the low magnifying power they are translucent, homogeneous, not granular, and circular in outline (Fig. 80).

Decolorized by Gram's method. Not motile. Will not grow without oxygen.

FIG. 80.—Bacillus of influenza; colonies on blood agar (F. T. Lord; photo. by L. S. Brown).

Pathogenesis.—The ordinary laboratory animals are not susceptible to infection with this organism.

Occurrence.—Found in the exudate of the respiratory tract in influenza, frequently inside of leucocytes (Fig. 79). It may be present in the small bronchi and in the exudate of broncho-pneumonia in this disease. It has been observed in purulent meningitis secondary to influenza.

F. T. Lord, working in the Laboratory of the Massachusetts General Hospital, found influenza bacilli in 60 of 100 unselected specimens of sputa repeatedly negative for tubercle bacilli. In 29 of these 60 cases the bacilli were present in great numbers. Eleven cases were of acute and 18 of chronic inflammation of the respiratory tract. In the chronic cases he demonstrated the persistence of influenza bacilli in the sputum for months or years.

Lord believes that influenza bacilli are very commonly present in sputa apart from epidemics of influenza, and that chronic infection with influenza bacilli is not infrequently mistaken clinically for tuberculosis.

Diagnosis.—Microscopical examination of cover-glass preparations of the bronchial sputum shows very small, short, round-ended bacilli, often in very large numbers and frequently in the pus-cells. These bacilli frequently occur in pairs and resemble pairs of cocci. Their ends may be more deeply stained than the central portions. For the staining of cover-glass preparations of the sputum Pfeiffer recommends that a very dilute carbol-fuchsin solution be applied for five to ten minutes. The cover-glass preparation is to be made from a distinctly purulent portion of the sputum. Staining with Löffler's methylene-blue solution also gives good results. See also W. H. Smith's method for staining the capsule of the pneumococcus, page 482.

The bacillus of influenza may be cultivated from the sputum by breaking up a small portion of a distinctly purulent character in 1 or 2 c.c. of bouillon, and then spreading a platinum loopful of the suspension over the surface of a blood-agar-agar slant, which is then placed in the incubator. After eighteen to twenty-four hours the characteristic colonies may be visible with the aid of a hand-lens. These should not grow in ordinary media unless blood or hemoglobin be present, and should have the morphology of the bacillus of influenza.

F. T. Lord obtains the best results by using as a culture-medium 1 part sterile horse-blood and 2 parts nutrient agar-agar in "slant" tubes. The blood is mixed with fluid agar-agar at 40° C. The colonies of the influenza bacilli may attain a diameter of more than a millimeter on this medium. The horse-blood may be easily obtained from any antitoxin plant and may be kept on hand in test-tubes for months without impairment of its utility for cultural purposes.

Bacillus Pertussis (**Bordet-Gengou**).—The bacillus of whooping-cough was discovered by Bordet and Gengou in 1900 in the sputum of acute cases of the disease. The first cultures were obtained by them in 1906.

Morphology.—The micro-organism is an extremely small non-motile bacillus. Frequently it is so short that it resembles a micrococcus. It is more regularly ovoid and somewhat larger than the influenza bacillus, and shows less tendency to pleomorphism and involution. It stains readily with

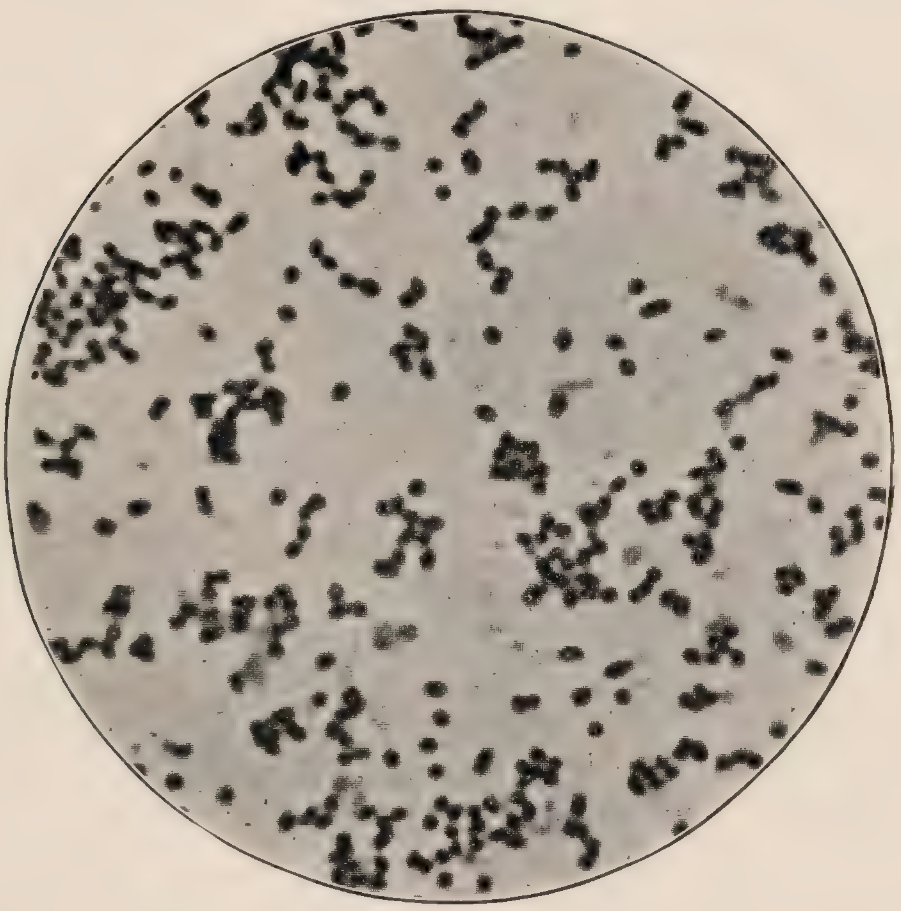


FIG. 81.—Bacillus pertussis from a culture; × 2000 (Mallory).

the ordinary aniline dyes, and usually more intensely at the ends than in the middle. It is decolorized by Gram's method.

Cultivation.—It is difficult to start a growth of the whooping-cough bacillus, although the organism grows readily enough after it has become accustomed to an artificial medium. Bordet and Gengou obtained their first cultures on a special potato-blood-agar medium which they devised and which remains to-day the only medium on which a growth can be started.

The medium is prepared as follows:

(*a*) A glycerin extract of potato is prepared by steaming in an autoclave 100 grams of sliced potato in 200 c.c. of a 4 per cent. solution of glycerin in water.

(*b*) To 50 c.c. of this extract add 150 c.c. of a 0.6 per cent. salt solution and 5 grams of agar. Melt the mixture in the autoclave, pour 2 to 3 c.c. into test-tubes, and sterilize.

(*c*) After sterilization add an equal volume of sterile defibrinated rabbit blood, or, preferably, human blood, mix the two fluids, slant the tubes, and solidify the mixture.

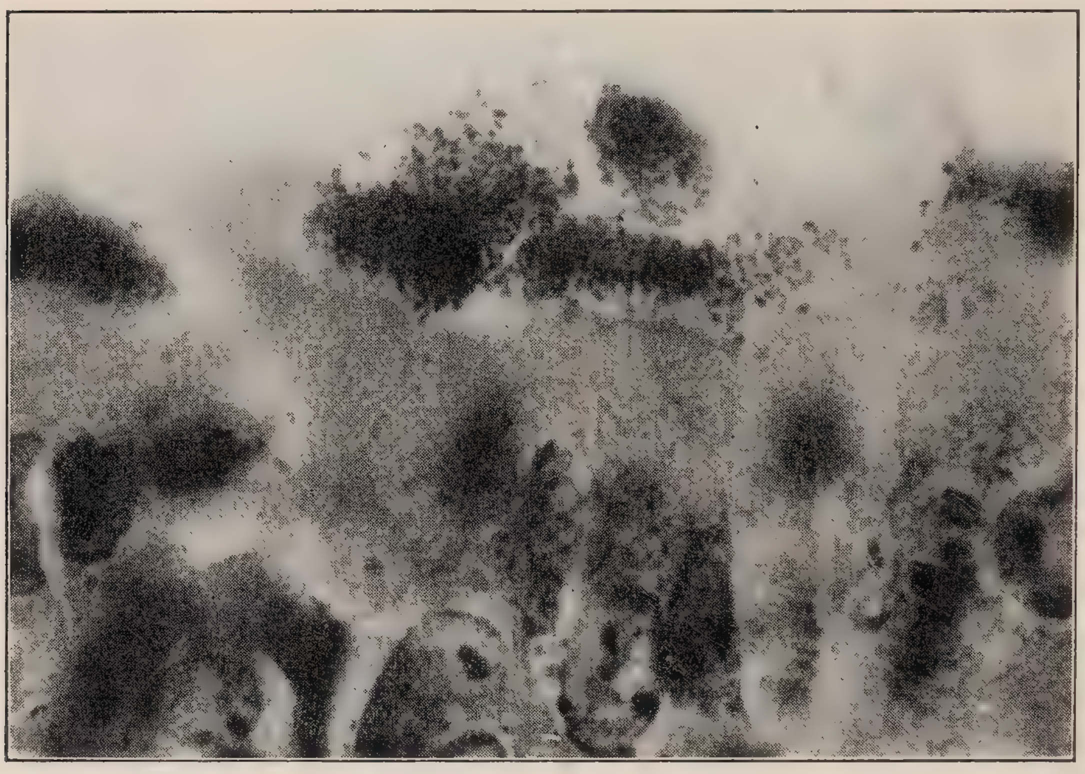


FIG. 82.—Bacillus pertussis in masses between the cilia of the epithelial cells lining the trachea; × about 2000 (Mallory).

Cultures should be made from the sputum during the early days of the disease. The organisms grow slowly at first, so that the colonies are barely visible after twenty-four hours. After forty-eight hours they are plainly visible as small, grayish, rather thick colonies. Later generations grow with luxuriance. After they have been cultivated for some time on the potato-blood-agar medium they will grow readily on plain blood-agar or ascitic-agar and in ascitic broth, or broth to which blood has been added.

Occurrence.—The bacillus of whooping-cough is found in large numbers and in practically pure culture in the early

stages of the infection. It occurs free in the secretion, and also within polymorphonuclear leucocytes. Study of post-mortem cases has shown that the organism is located between the cilia of the epithelial cells lining the trachea and bronchi. This location seems to be characteristic for this organism in man.

In experimental work with this bacillus on animals, especially puppies and young rabbits, it is necessary to bear in mind that the Bacillus bronchisepticus, the cause of distemper, snuffles, etc., in animals, is often present, and that it is of the same size and occupies the same peculiar position between the cilia of the cells lining the air-passages, including the nasal cavity. It differs from the bacillus of whooping-cough in being motile and in producing alkali in litmus-milk.

Bacillus of Glanders (Bacillus Mallei).—*Blood-serum.*—Rounded, elevated, colorless, viscid-looking colonies, growing slowly and becoming well developed after thirty-six hours in the incubator. They may attain a diameter of 2 or 3 mm., and after a time they assume a brownish tint.

FIG. 83.—Glanders bacilli from a young culture on potato; × 2000 (Wright and Brown).

Morphology.—Bacilli of medium size, variable in length, having round or conical ends, and frequently showing faintly stained areas in their protoplasm (Fig. 83). The larger forms of the bacillus are usually slightly bent or wavy in outline.

Slight irregularities in shape may be observed. The morphology varies considerably on different culture-media.

In cover-glass preparations from the lesions the bacilli usually appear somewhat longer and thicker than the tubercle bacillus, and show numerous sharply defined, unstained or faintly stained areas in their protoplasm (Fig. 84). They have rounded or conical ends, and are sometimes slightly irregular in shape. As a rule, they are present in small numbers. If Löffler's methylene-blue solution is used for staining the cover-glass, it should be heated; if carbol-fuchsin is used, it should be followed by a slight decolorization with 95 per cent. alcohol to better differentiate the bacilli.

Potato.—After thirty-six hours in the incubator a rather thick, colorless, viscid-looking layer appears, which soon

FIG. 84.—Glanders bacilli in a cover-glass preparation from a lesion in a guinea-pig, showing the marked irregularity in the staining of the bacilli; × 2000 (Wright and Brown).

assumes a brownish tint and resembles honey in appearance. Later the brown color changes to a dark reddish-brown, and the growth becomes thicker and more opaque, while the potato takes on a dark-gray color.

Pathogenesis.—When inoculated subcutaneously into guinea-pigs, the characteristic results are swelling and inflammation of the scrotum, appearing after a variable number of days, often after a week.

The animals usually survive several weeks, with ulceration at the point of inoculation. The lesions produced consist

in suppurative processes or abscess-formations in or about the testes, in the lymph-glands, in the anterior nares, about the joints, and in other situations, besides small grayish nodules or areas in the viscera—the so-called "glanders tubercles." The suprarenal capsules usually show red areas, and they may be enlarged. On microscopical examination the small nodules as well as the extensive suppurative areas will be found to be composed of necrotic material containing leucocytes and fragments of chromatin. The distribution and extent of the lesions vary with each animal, but the involvement of the testis or its membranes is practically constant and pathognomonic of the bacillus of glanders. This involvement of the testis may consist, in early cases, in the presence of yellow foci in or about the tunica vaginalis, or in later cases the organ may show large yellow areas with purulent softening.

Intraperitoneal inoculation with virulent cultures may be followed by death within forty-eight hours, with fibrinous exudate on the peritoneum in which minute grayish nodules are seen. The nodules are made up of a material which is apparently mainly dead or degenerated leucocytes and desquamated peritoneal endothelium, together with many chromatin fragments.

In these acute cases also microscopical examination of the spleen and liver may show the presence of small nodules identical in structure with those seen in the more chronic cases. For the purpose of producing with cultures the characteristic lesions of the testis or its coverings it is better to inoculate the animal subcutaneously, for in the rapidly fatal intraperitoneal inoculations with virulent cultures these may not show any marked changes.

The bacilli may be cultivated from the lesions, but not from the blood of the heart, in the chronic cases. They may be present in the blood of the heart, however, in small numbers in rapidly fatal infections following intraperitoneal inoculation.

Field-mice may die from subcutaneous inoculation in about seventy-two hours. The most conspicuous lesion pro-

duced is enlargement of the spleen, with the presence in it of minute grayish nodules. White mice are immune. Rabbits are not so susceptible as guinea-pigs to the infection.

Decolorized by Gram's method. Not motile. Spore-formation not probable. Rate of growth is slow.

Bouillon.—Diffusely clouded, with the formation of a viscid sediment.

Litmus-milk.—Gradually turned red and coagulated.

Agar-agar and Gelatin.—Growth not especially characteristic.

Occurrence.—Found in the lesions of glanders and of farcy, and may invade the blood-stream in small numbers in acute cases of infection. Grows in the tissues in clumps or groups as well as scattered. In lesions on exposed surfaces it may be accompanied by the pyogenic cocci. We have succeeded in demonstrating the presence of the bacillus in the sputum of a case of human glanders by inoculation of a guinea-pig with the sputum.

Diagnosis.—In a case of suspected glanders the discharges from sinuses or ulcerated surfaces, or the contents of pustules, are to be examined for the presence of the bacillus of glanders by the usual methods.

The material for examination may be collected on "swabs." With this a guinea-pig is to be inoculated and cultures and cover-glass preparations are made. If the material be from sinuses or ulcerated surfaces, the isolation of the bacillus by cultures will be difficult, owing to the presence of other organisms. The guinea-pig is to be inoculated in the peritoneal cavity by introducing the infected swab into it through an incision in the abdominal wall, or by injecting about 1 c.c. of a suspension in bouillon of the suspected material into the peritoneal cavity with a hypodermic syringe.

If the bacillus of glanders is present, the scrotum will usually show the characteristic swelling and inflammation in the course of three or four days, and death will occur after some weeks. In some cases the animal may die in thirty-six or forty-eight hours. In any case the characteristic lesions of glanders will be found as described elsewhere,

and the bacillus may be isolated from them by cultures. The spleen will practically always yield glanders bacilli in pure culture even if no macroscopical lesion can be made out.

In cultures the organism should show those characteristics of morphology, of culture, and of pathogenesis which have been described above.

Bacillus of Chancroid (**Bacillus of Ducrey**).—In smears from the lesions the bacilli appear as short, round-ended rods, about 1.5 μ long and 0.5 μ thick, occurring characteristically but not always in chains. The middle portion of the rods does not stain so deeply as the ends. The bacilli are decolorized by Gram's method of staining, and are not motile. The following description of the cul-

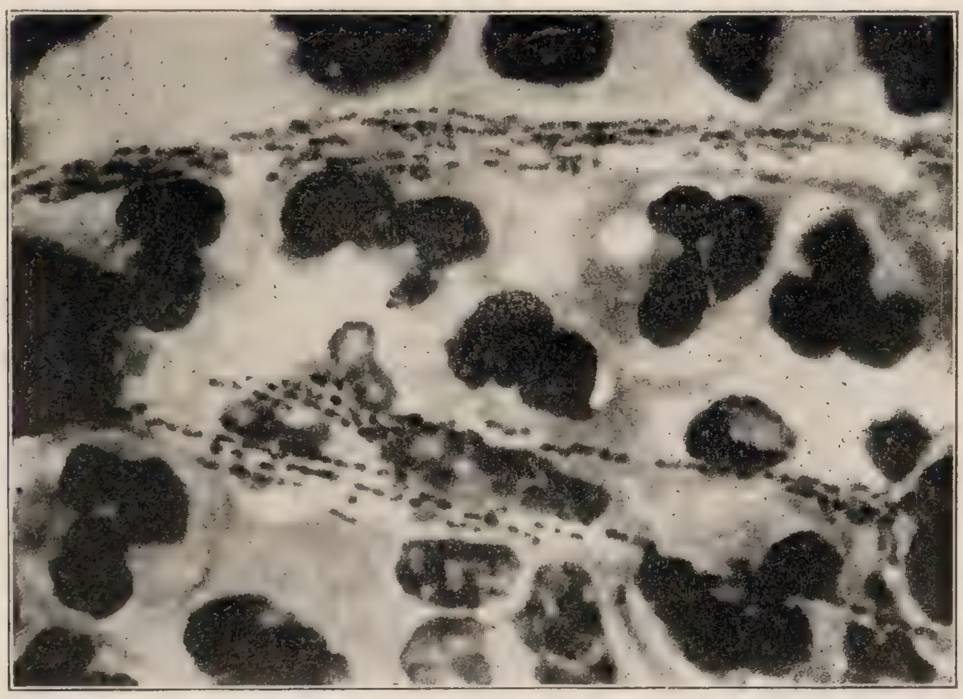


FIG. 85.—Bacillus of chancroid in smear preparation from pus (Lincoln Davis; photo by L. S. Brown).

tural peculiarities of the chancroid bacillus are based on the observations of Dr. Lincoln Davis in the Laboratory of the Massachusetts General Hospital.

The bacillus does not grow on the ordinary culture-media, but may be cultivated in blood or in media containing one-third its volume of blood. It is essential that the blood be fresh. In tubes containing blood, or a mixture of bouillon and blood, after twenty-four hours in the incubator, the growth appears as whitish flocculi at the bottom of the

tube. These flocculi are composed of tangled chains of the bacilli, the chains being often of extreme length. The individual bacilli, as a rule, have the same morphology and staining reactions as in the smears from lesions, but occasionally long or even filamentous forms may be seen. On the surface of slant tubes, composed of a mixture of fresh blood and agar, the bacillus forms, after forty-eight hours in the incubator, small, rounded, grayish colonies difficult to pick up with the platinum wire, because they tend to glide before it. The bacilli from these colonies appear in smear preparations in short chains and singly. Involution forms are early apparent among the bacilli in all cultures. The bacillus dies out in cultures after about three days. Upon a monkey of the genus *Macaccus*, small ulcerations in the skin were produced by inoculation with cultures.

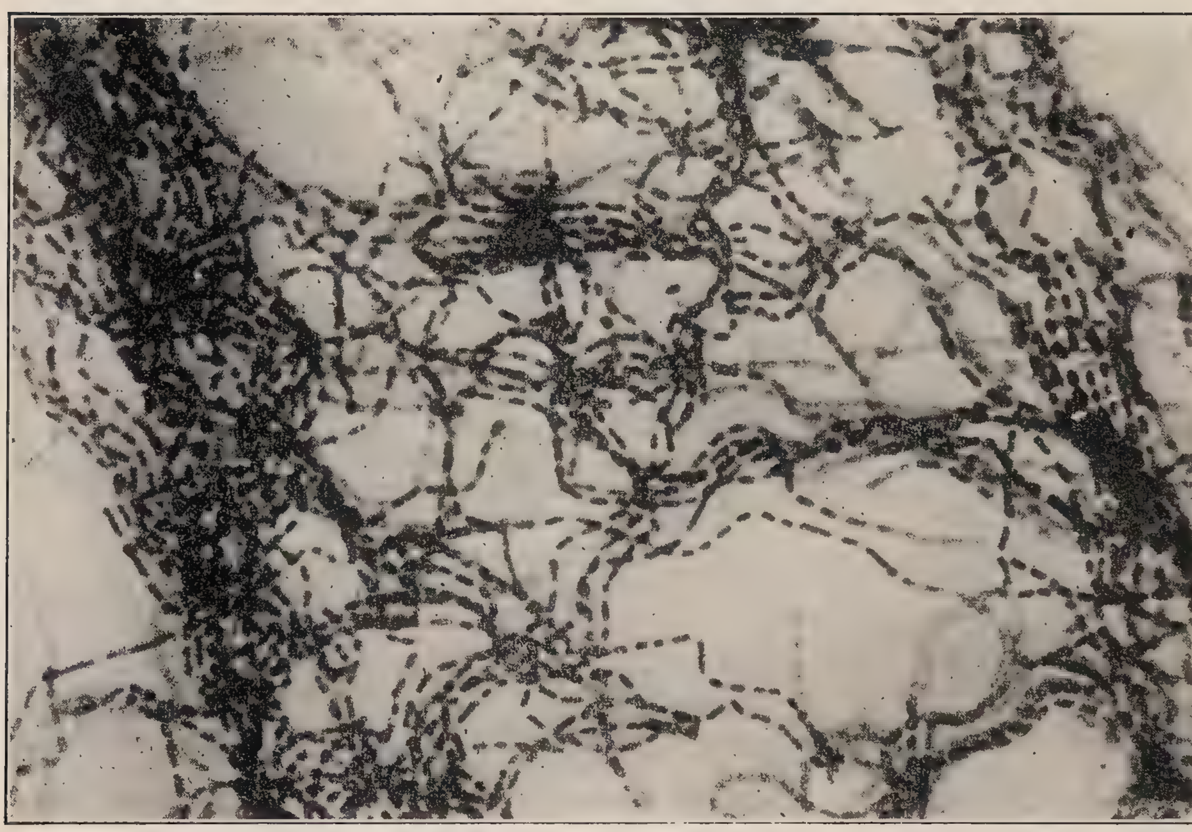


FIG. 86.—Bacillus of chancroid from culture (Lincoln Davis; photo by L. S. Brown).

Method of Isolation from the Lesions.—Dr. Davis found that pure cultures were readily obtained by inoculating a small quantity of freshly drawn human blood in small tubes with material from the lesions, the fresh blood apparently inhibiting or destroying other bacteria. These small tubes

containing blood are most easily prepared in a way devised by J. H. Wright. A small glass tube, about 5 or 6 cm. long and 4 or 5 mm. in internal diameter, is drawn out into a fine caliber at one end, and is then sterilized throughout in the gas-flame. When cool, the pointed extremity of the tube is immersed in the blood obtained from a needle-prick in the skin of the dorsum of the thumb near the nail, and then by manipulation of the tube the blood is caused to flow into it. In this way a sufficient quantity—say, 0.2 to 0.5 c.c.—is easily collected in the tube, after which the pointed end is sealed in the flame and the tube is ready to be inoculated. The other end of the tube is plugged with cotton, which is impregnated with paraffin to prevent evaporation. The skin, before being pricked, is sufficiently cleansed by soap and water, followed by alcohol. A small tourniquet is applied about the base of the thumb, to increase the flow of blood from the needle-prick.

Bacillus Proteus (Proteus Vulgaris).—*Morphology.*—Bacilli of very variable length, sometimes appearing like cocci or as filaments.

Motile, being provided with terminal flagella. Does not stain by Gram's method.

Colonies in Gelatin.—Rapid growth with liquefaction of the gelatin. In a medium containing 5 per cent., instead of 10 per cent., of gelatin prolongations from the margins of the colonies may be formed. These may become separated from the mother colonies and form daughter colonies. Motions may be observed in these prolongations.

Gelatin Stab.—Rapid liquefaction along the line of inoculation with cloudiness of the liquefied gelatin and a flocculent deposit.

Agar-agar Slant.—Widely spreading, thin, moist, grayish-white layer.

Potato.—Dirty white, moist layer.

Litmus-milk.—Turned pink and slowly coagulated.

Odor.—The cultures generally have a putrefactive odor.

Pathogenesis.—Intravenous, intraperitoneal, or intramuscular inoculations of rabbits may produce death in twenty-

four to thirty-six hours after moderately large doses. Liquefied gelatin-cultures are said to be more virulent than bouillon cultures. Guinea-pigs seem to be less susceptible than rabbits to infection with this organism.

Occurrence.—This bacillus and its varieties are among the most common and widely distributed putrefactive bacteria. It occurs in the intestinal contents. In pathological examinations it may be found in peritonitis and in abscesses, usually associated with other bacteria. It may also invade the circulating blood.

The so-called "proteus group" includes several varieties of similar organisms—viz. the *proteus vulgaris*, the *proteus mirabilis*, and the *proteus Zenkeri*. The latter does not liquefy the gelatin, while the *proteus mirabilis* liquefies it slowly.

Bacillus Mucosus Capsulatus.—*Blood-serum.*—After twenty-four to thirty-six hours in the incubator the colonies

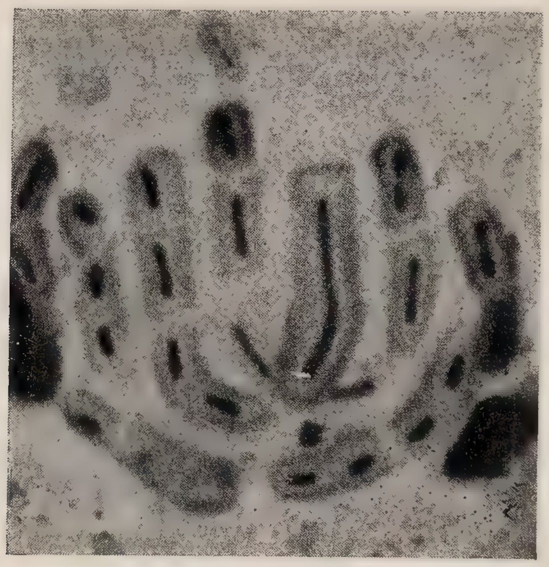


FIG. 87.—Bacillus mucosus capsulatus; cover-glass preparation from sputum. Stained by W. H. Smith's method; × 1500 (W. H. Smith; photo. by L. S. Brown).

appear as translucent, colorless, rounded, convex elevations, resembling drops of mucus. If few in number, they may attain a diameter of 2–3 mm. They are viscid, and when touched with the platinum wire may be drawn out into threads. The water of condensation may become thick or viscid from the growth of the organism in it.

Morphology.—Bacilli of moderate size, usually two or three times as long as broad, with rounded ends, occurring

frequently in pairs and sometimes in long forms. Occasionally in cultures it shows a wide capsule. The capsule, however, is best shown in cover-glass preparations from infected tissues (Figs. 87, 89).

Pathogenesis.—White mice, rabbits, and guinea-pigs die from septicemia in a short time after inoculation, the capsule bacilli being present in the organs and blood of the heart in large numbers.

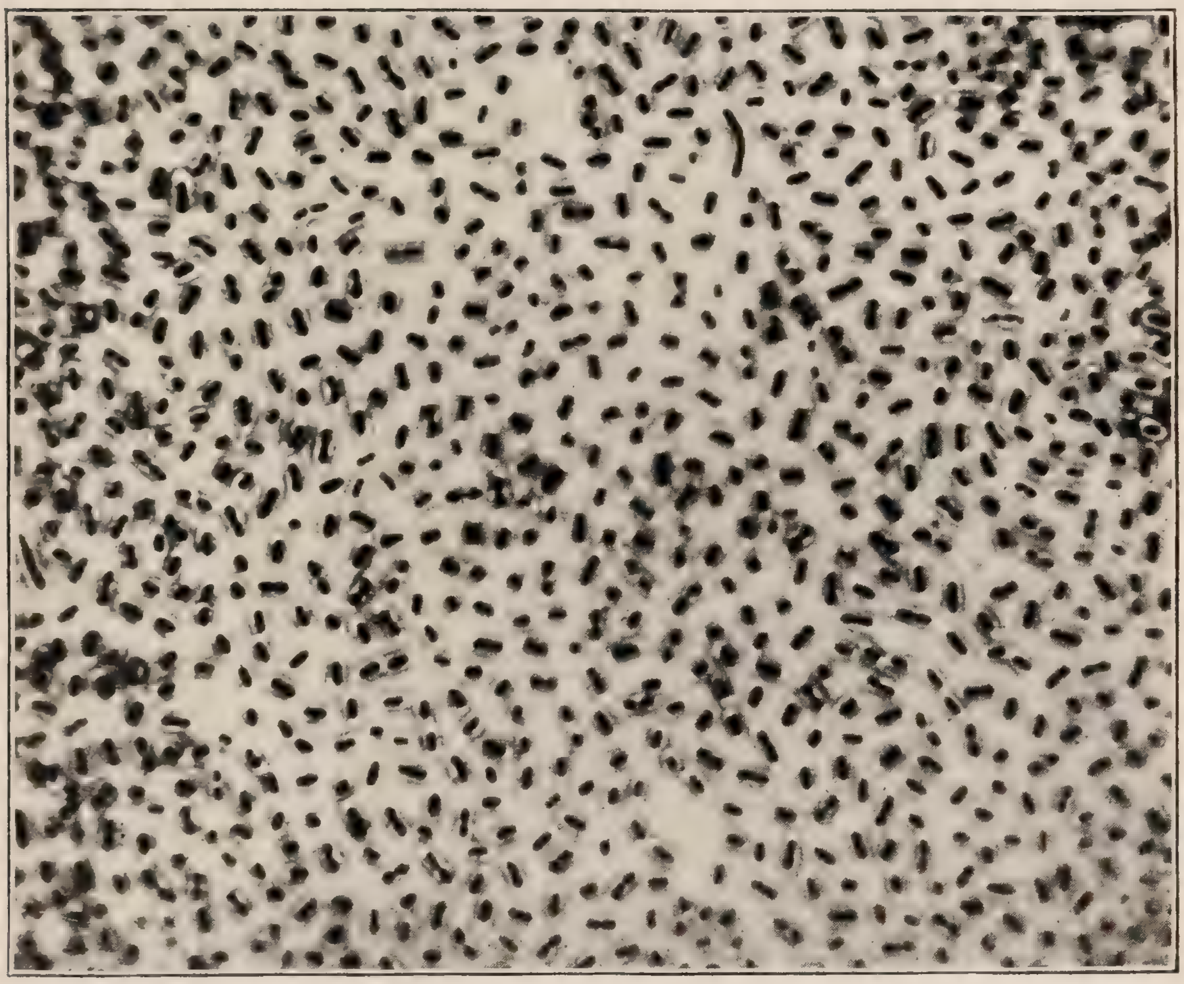


FIG. 88.—Bacillus mucosus capsulatus, from a culture; × 1000 (Wright and Brown).

White mice die in twenty-four hours to three days. Rabbits inoculated in the ear-vein and guinea-pigs inoculated in the peritoneal cavity may die within twenty-four hours.

Subcutaneous inoculation of the animals last named leads only to local suppuration. The lesions produced by this organism consist in marked congestion of the superficial veins, hemorrhage into the lymphatic glands, and enlargement and softening of the spleen. In the guinea-pig a hemorrhagic condition of the supra-renal capsules is present, and in the peritoneal cavity there may be a small amount

of clear, rather viscid fluid containing the bacilli in large numbers.

The organs on microscopic examination may show peculiar areas in which the cells and nuclei are shrunken and in which the bacilli are aggregated.

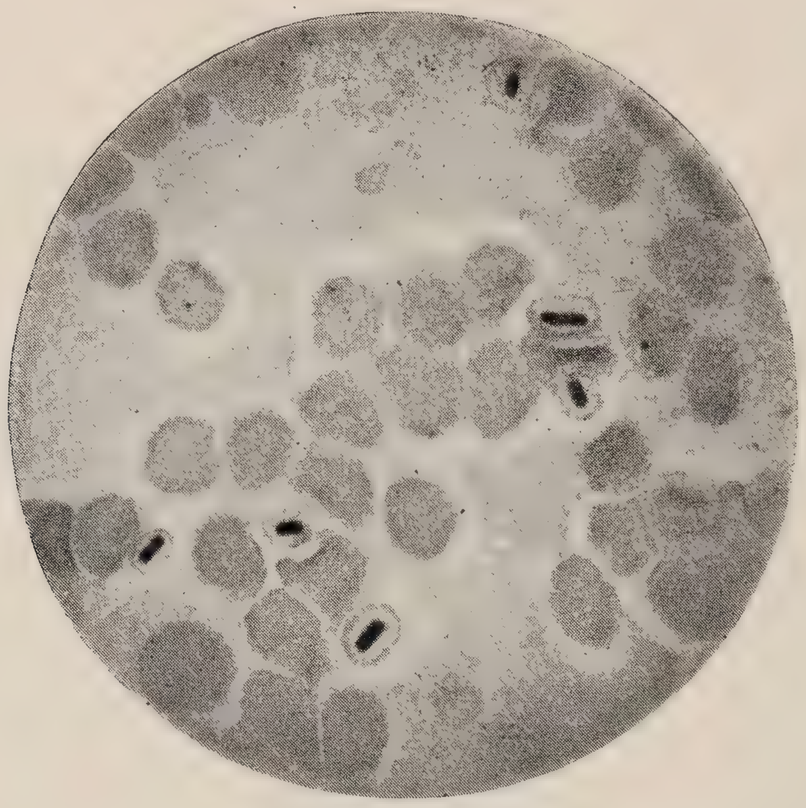


FIG. 89.—Bacillus mucosus capsulatus in blood; × 1000 (Fränkel and Pfeiffer).

Decolorized by Gram's method. Not motile. Does not form spores.

Glucose Agar-agar Stab.—Growth along the line of inoculation, with the production of a few gas-bubbles in the medium.

Bouillon.—Clouded with the formation of a thin pellicle.

Potato.—Thin, colorless, viscid layer.

Litmus-milk.—Turned red and coagulated.

Gelatin.—Growth not remarkable.

There apparently exists a number of varieties of aërobic capsulated bacilli differing from one another only in non-essential particulars. The organism here described is to be taken as a type of a group of closely-related bacteria of which the *bacillus pneumoniæ of Friedländer* is a well-known member.

Occurrence.—This organism or closely related forms may be met with in broncho- or lobular pneumonia and in inflammatory conditions of the air-passages generally. It may also be present in the upper air-passages of healthy individ-

uals. It has been observed in inflammations of the middle ear, in empyema, meningitis, endocarditis, peritonitis, and in pus-formations. In fatal infections the blood-stream may be found invaded by the organism. It is held by some bacteriologists that the members of this group may be the infective agents in genuine croupous pneumonia in rare instances. Representatives of this group have been found in the soil, in the air, and in contaminated water.

Bacillus of Tetanus.—This bacillus will not grow in the presence of oxygen.

FIG. 90.—Tetanus bacilli showing flagella, from a preparation stained by Dr. Hugh Williams; × 2000 (Wright and Brown).

Morphology.—Slender rods with rounded ends, which may grow into long threads. In the incubator spores are rapidly formed. These are round, wider than the bacillus, and are situated at the end of the rod, giving the appearance of a drum-stick or a round-headed pin (Fig. 94).

The *colonies* in anaërobic glucose-gelatin cultures appear after several days as small clumps of interlacing fibrillæ from which delicate filaments radiate into the gelatin, which is slowly liquefied.

The colonies in simple anaërobic glucose-agar plate cultures (see page 126) appear after twenty-four to forty-eight hours in the incubator, as groups and masses of long filaments radiating from a center (Fig. 93).

Pathogenesis.—Subcutaneous inoculation of mice at the root of the tail gives rise to tetanic symptoms in twenty-four hours, followed by death in two or three days.

Guinea-pigs and rabbits are also susceptible to the infection, the period of incubation in these animals being twenty-four to thirty hours in the former and two to three days in the latter animal, after subcutaneous inoculation. The symptoms of tetanus appear first in the extremities nearest the

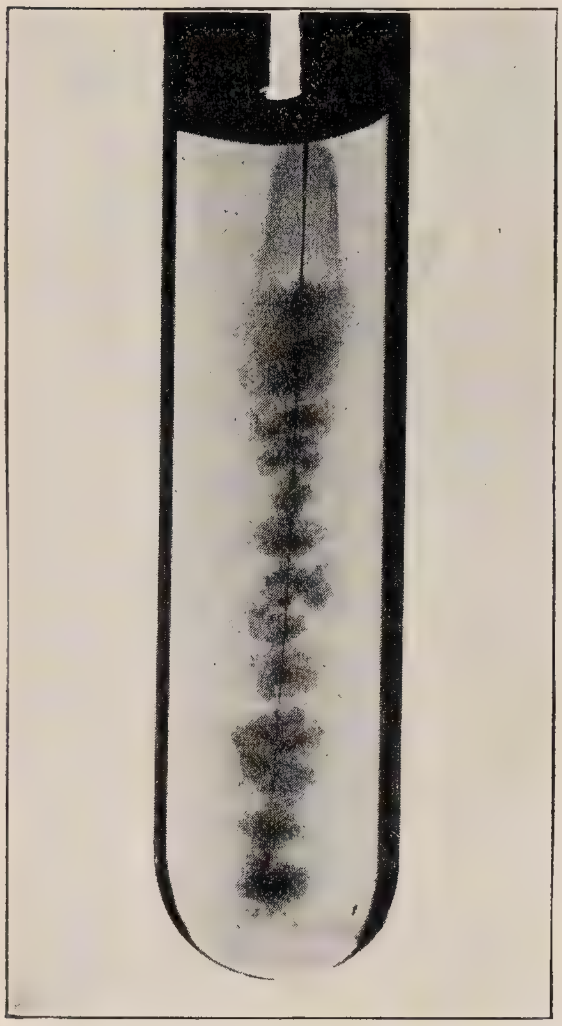


FIG. 91.—Tetanus bacillus. Stab-culture in glucose-agar. In the upper layers of the medium the peculiar brownish coloration is shown.

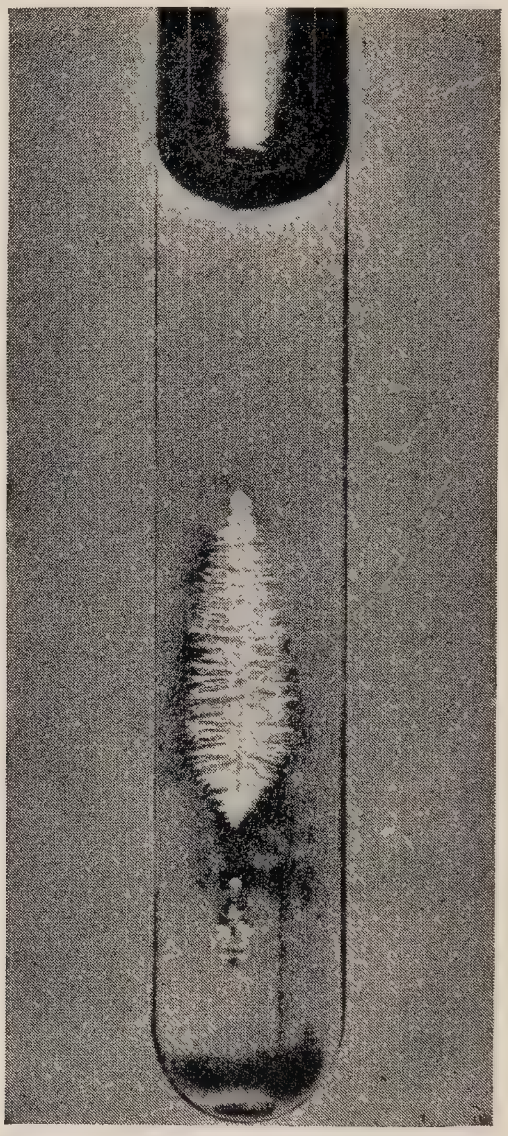


FIG. 92.—Bacillus of tetanus: six-days-old stab-culture in glucose-gelatin (Fränkel and Pfeiffer).

point of inoculation. In mice the hind legs become rigidly extended backward. At the autopsy the bacillus is to be found only at the point of inoculation, and may be difficult or impossible to demonstrate there.

Glucose-gelatin Stab.—Growth along the line of inoculation, beginning 2 or 3 cm. below the surface, with delicate

filaments radiating laterally into the gelatin (Fig. 92). Liquefaction and gas-production occur.

In *deep-stab cultures in glucose-agar* faintly alkaline to litmus (see Fig. 91) growth appears first all along the line of inoculation to within about 1 cm. of the surface after about twenty-four hours in the incubator. Later, lateral outgrowths extend into the medium from all along the line of

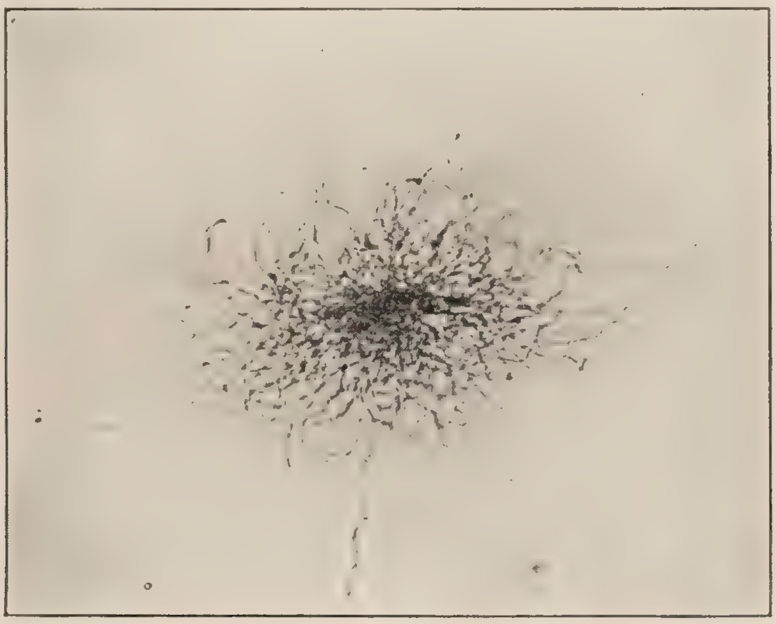


FIG. 93.—Colony of tetanus bacilli in anaërobic glucose-agar plate; low magnifying power (Wright and Brown).

inoculation below a point about 1 cm. below the surface. In the portion of the line of inoculation above this, growth is frequently observed up to the surface, but without lateral outgrowths. The growth eventually assumes the appearance of an inverted pine tree. A peculiar feature of the culture is the appearance of a brown pigmentation in the culture-medium in its upper layers in the form of a flat or cone-shaped zone. A small quantity of gas may be produced.

If the agar has a reaction of about 1 per cent. normal acidity to phenolphthalein (see p. 85) growth appears along the line of inoculation and spreads through the medium as a cloudiness extending to within a few millimeters of the surface. The employment of glucose culture-media not older than a week or so seems to be important for success in cultivating this organism.

In the vegetative form the organism is sluggishly motile. It has numerous flagella. It is stained by Gram's method.

Glucose-bouillon.—Growth appears first, after twenty-four to forty-eight hours, as a diffuse cloudiness. Later the fluid becomes clear, and a grayish sediment collects at the bottom of the tube. Only a small amount of gas is produced.

Occurrence.—Found in the soil, and often in the feces of herbivorous animals. In cases of tetanus the bacillus is to be found only in the wound or at the point of inoculation. It does not invade the blood-current.

The bacillus of tetanus acts by the production of a "*toxin*" or "*toxalbumin.*" This is also produced in cultures. It may be demonstrated in the bacteria-free filtrate of bouillon cultures some days or weeks old. A very few drops of this fluid will give rise to fatal tetanus in a mouse.

Method of Isolation.—Tetanus bacilli will grow in aërobic culture if other bacteria are growing with them. Since tetanus wounds usually contain other bacteria, all that is necessary to obtain an impure culture of the tetanus bacillus is to inoculate an ordinary blood-serum culture-tube (see page 80) with material from the wound. After several days or a week in the incubator, if tetanus bacilli are present they can be recognized by cover-glass preparations from the growth in the tube by their morphology and spore-formation (see Fig. 94). There will also be a peculiar, stinking odor about the culture. The isolation of the tetanus bacillus is now to be proceeded with as follows: Mix a loopful of the mixed growth on blood-serum with a tube of sterile bouillon, and heat in a water-bath for at least fifteen minutes at 80° C., then make anaërobic cultures from this (see Anaërobic Methods, page 123), taking several loopfuls for inoculation.

If other spore-bearing bacilli are present in the mixed culture in the blood-serum tube, it will be necessary to use some form of anaërobic culture on a solid medium in order to obtain separate colonies of the tetanus bacillus for further cultures.

The bacillus may be isolated from wounds and from the soil by inoculation of mice subcutaneously, and proceeding as above described with material from the seat of inoculation.

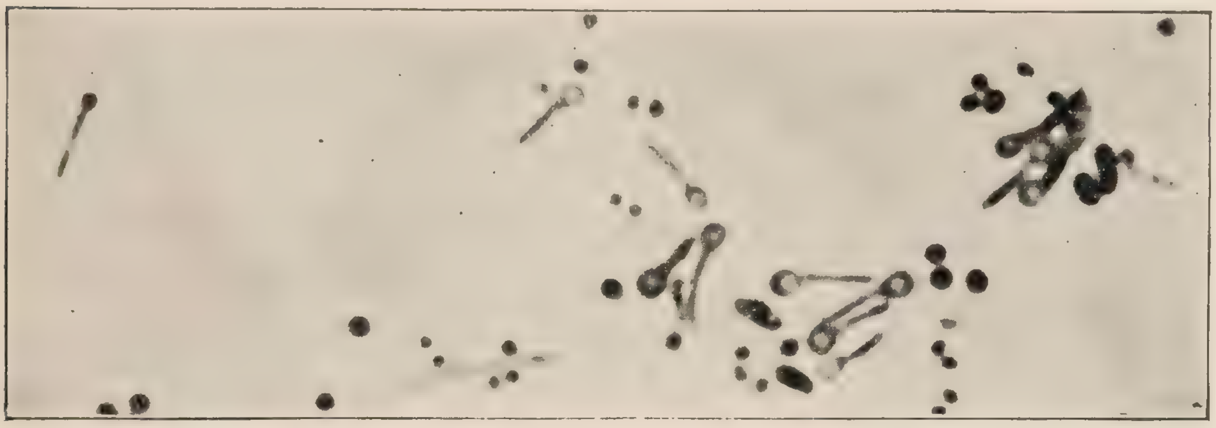


FIG. 94.—Spore-bearing tetanus bacilli in an impure culture on blood-serum from a case of tetanus. In the bacillus on the extreme left the beginning of spore-formation is shown (Wright and Brown).

Bacillus Aërogenes Capsulatus.—Will not grow in the presence of oxygen.

Morphology.—Bacilli of about the thickness of the anthrax bacillus, variable in length, but usually 3 to 6μ long. Ends rounded or square cut. Occurs singly, in pairs, in clumps, and sometimes in short chains, less frequently in threads and long chains.

May show unstained spots or deeply staining granules in the protoplasm. Capsules may be frequently demonstrated in the specimens from the tissues, and sometimes in agar-agar cultures.

Colonies in anaërobic cultures are grayish to brownish-white, with a central darker spot by transmitted light. In time they may attain a diameter of 2 to 3 mm. or more. Colonies in the depths are spherical or oval, sometimes presenting knob-like or feathery projections.

Effects on Animal Tissues.—Not pathogenic for rabbits.

If a rabbit that has received 0.5 to 1 c.c. of a bouillon culture injected into the ear-vein be killed immediately afterward and the body kept for twenty-four hours at a temperature of 18° to 20° C., or for four to six hours at a temperature of 30° to 35° C., the vessels and organs will be found to contain a great quantity of gas and large numbers of

the bacilli. The organism multiplies post-mortem in the blood of the animal and produces the gas. This effect upon the tissues of the dead animal is characteristic of the bacillus.

The subcutaneous inoculation of guinea-pigs with young cultures may produce fatal gas phlegmons. The hemorrhagic fluid from the dead animal is virulent for other guinea-pigs, and may be virulent for rabbits.

FIG. 95.—Bacillus aërogenes capsulatus; cover-glass preparation from the spleen. Stained by W. H. Smith's method; × 1500 (W. H. Smith; photo. by L. S. Brown).

Gas-production is marked in agar-agar and gelatin cultures containing glucose. The gas produced burns with a blue flame and is odorless.

Gelatin is liquefied slowly and to a limited extent.

Glucose Bouillon.—Diffusely clouded at first, later becoming clearer, with an abundant whitish, more or less viscid sediment.

Milk.—Coagulated, the clot being firm, retracted, and furrowed with the marks of gas-bubbles.

Potato.—Growth thin, moist, and grayish-white, or it may not be visible.

The bacillus is stained by Gram's method. It is not motile. Spore-formation has been observed by E. K. Dunham.

The vitality of the organism depends upon the character of the culture-medium and the mode of cultivation. It survives longer when cultivated by Buchner's method (see page 126) than when cultivated under hydrogen. Cultures on glucose media are shorter lived than those on plain media.

Occurrence.—Occurs at autopsies in which gas-bubbles are present in the larger vessels, accompanied by the formation of numerous small cavities in the liver containing gas. It has been found also in emphysematous phlegmons, in puerperal sepsis, in peritonitis, and in other conditions.

Bacillus of Malignant Edema.—This bacillus will not grow in the presence of oxygen.

Morphology.—Rather large bacilli, sometimes growing into threads (Fig. 96), but occurring frequently in pairs, in which the proximal ends are square while the distal ends are rounded. Forms oval spores in the middle of the rod, which may give the rod a spindle or oval shape.

The *colonies* in anaërobic glucose-gelatin cultures appear as spheres of cloudy liquefied gelatin marked by delicate radiating streaks. Gas-bubbles are formed in the medium (Fig. 97).

FIG. 96.—Bacillus of malignant edema from the edema fluid of a guinea-pig inoculated with garden-earth; × 1000 (Fränkel and Pfeiffer).

In glucose-agar the colonies appear as hazy points made up of interlacing filaments and resembling very much the colonies of the tetanus bacillus.

Pathogenesis.—Subcutaneous inoculation of mice, guinea-pigs, and rabbits is followed by death in from sixteen to forty-eight hours, depending upon the animal, mice being most susceptible. The typical lesions are extensive subcutaneous edema containing gas-bubbles and more or less blood, and enlargement of the spleen. The bacilli are found

in the edema, in the viscera, and on the serous surfaces of the organs, but not in the blood of the heart if the examination be made immediately after death, except sometimes in mice. The organism is not capable of multiplying in the living blood, owing to the presence of oxygen. In inoculating subcutaneously a deep pocket should be made in the skin, and the material for inoculation introduced into the tissue as far away from the opening as possible. This is to prevent the access of too much oxygen to the organism.

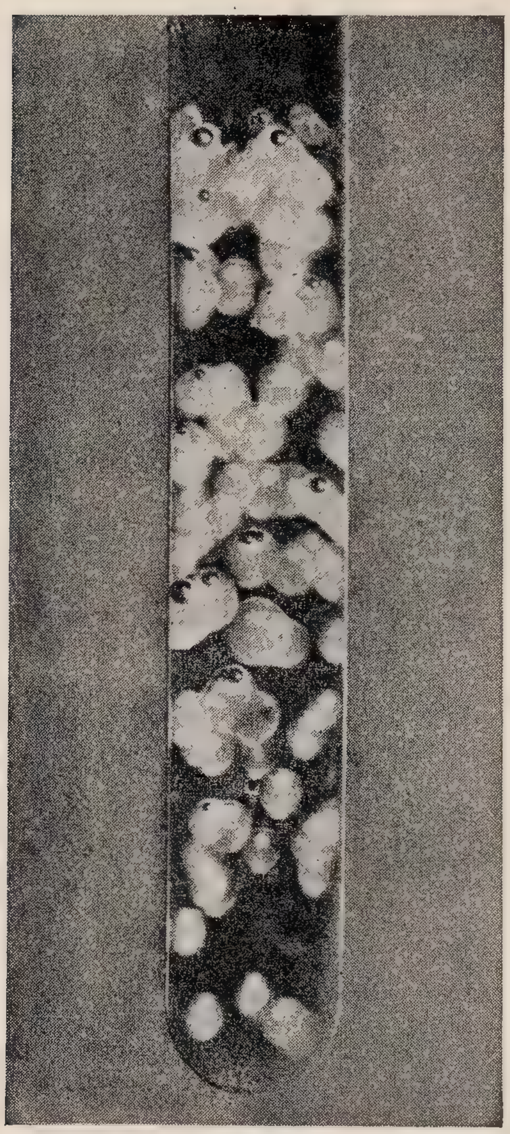


FIG. 97.—Bacillus of malignant edema; colonies growing in glucose-gelatin (Fränkel and Pfeiffer).

Slightly motile. Flagella may be demonstrated by special staining methods.

The bacilli in tissues are stained by Gram's method, but in cultures most of them are decolorized by it, probably because of rapid degenerative changes in them.

Growth in anaërobic agar-agar and bouillon culture is good, but not characteristic.

Occurrence.—Widely distributed in the soil and in putrefying substances. Only a very few cases are on record of infection in man by this bacillus.

The Micro-organism of Actinomycosis.—The proper name of this micro-organism is "Actinomyces bovis." It belongs to the group of filamentous branching micro-organisms which are regarded as occupying an intermediate position between the bacteria, on one hand, and the moulds or hyphomycetes on the other.

The organism appears in the pus from subacute or chronic suppurative lesions of the disease actinomycosis, as grayish

or yellowish granules, usually less than 1 mm. in diameter. Sometimes these granules are aggregated in groups of two

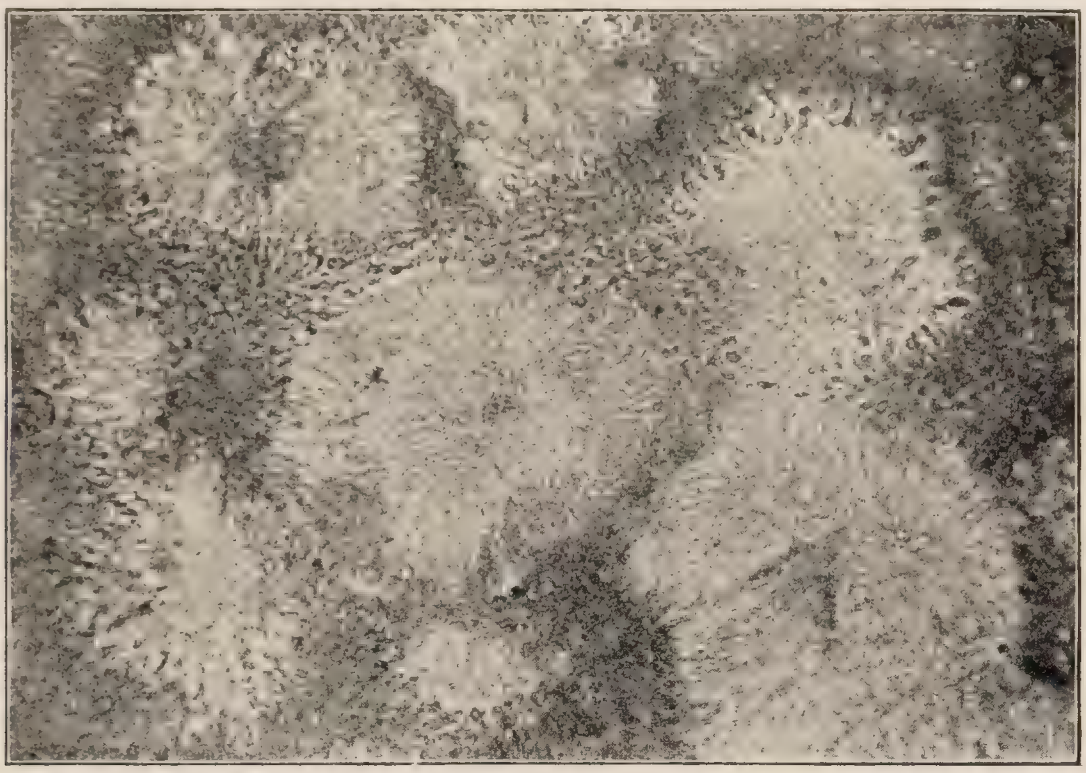


FIG. 98.—Actinomyces granule crushed beneath a cover-glass, showing radial striations in the hyaline masses. Preparation not stained; low magnifying power (Wright and Brown).

or three, and thus appear as lobulated larger granules. They are friable, and when gently crushed beneath a cover-glass and observed under the microscope, they are seen to have been broken up into hyaline rounded masses, at the

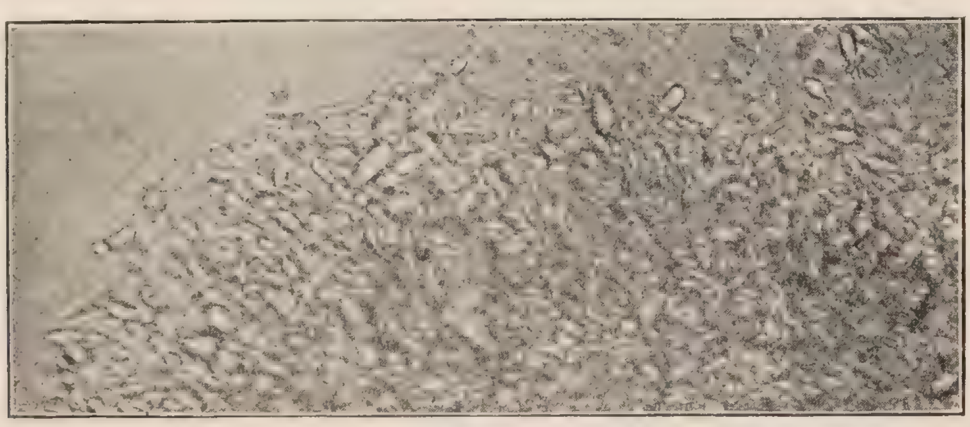


FIG. 99.—A portion of an actinomyces granule crushed beneath a cover-glass, showing the "clubs." The preparation not stained; moderately high magnifying power (Wright and Brown).

margins of which, on close inspection, fine radial striations or filaments or hyaline club-shaped bodies, all closely set together, may be seen (Figs. 98, 99). The club-shaped

bodies are variable in size, and are composed of a hyaline, refringent substance. The appearance of radial striation in the granule, when observed with the microscope, due to the presence and radial arrangement of these hyaline bodies, gave rise to the name "ray-fungus" for this parasite. Not all of the granules have these "clubs." In the granules obtained from the lesions in man they are much less frequently observed than in those obtained from the lesions in cattle.

If a cover-glass preparation be made by breaking up one of the granules and staining with Gram's method, there will usually be found, upon examination with an oil-immersion lens, isolated and matted filaments, many of which may be

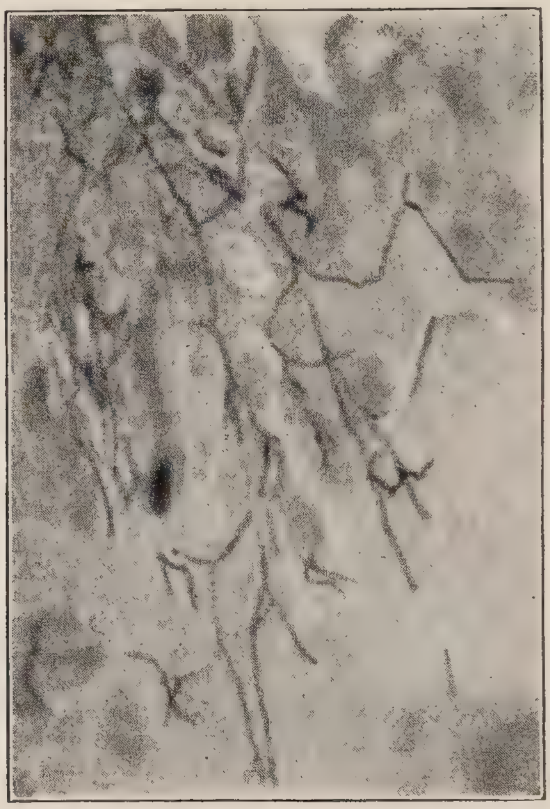


FIG. 100.—Branching actinomyces filaments in a cover-glass preparation made from an actinomyces granule stained by Gram's method; × 1000 (Wright and Brown).

seen to branch, in addition to longer and shorter fragments of filaments and fine detritus of the same (Fig. 100). The filaments are usually more or less wavy in their course, and are, as a rule, slightly thicker than the tubercle bacillus. Some of the filaments will be found to stain homogeneously; others do not stain so deeply, and show numerous deeply staining points in their substance. If clubs are present in

the granule, they also may be found scattered throughout the preparation.

In sections of the tissues stained by Gram's method two chief forms of granules are found. In one of these forms the granule is seen to consist of filaments embedded in a hyaline substance, and usually arranged at the periphery in an indefinite radiate manner (Fig. 101). At the margin

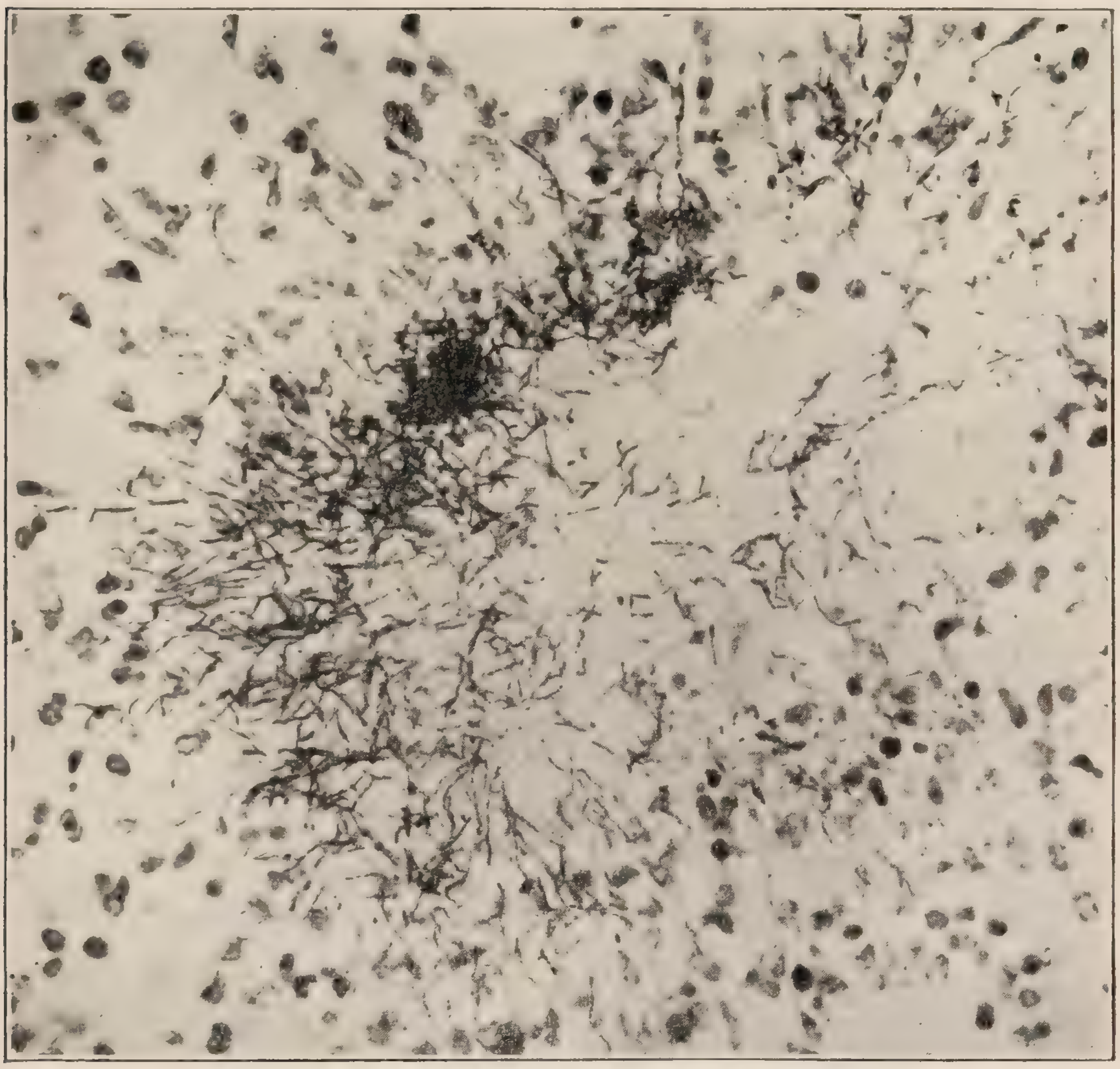


FIG. 101.—Colony or granule of actinomyces in a section through a lesion, showing the Gram-stained filaments and hyaline material and also the pus-cells surrounding the colony (Wright and Brown).

of the granule the filaments are usually much more numerous than in the central portions, where the hyaline material predominates. This hyaline material apparently consists of degenerate or dead filaments or their remains. The other form of granule seen in sections is distinguished by possessing at its margin a row of closely set radiating club-shaped bodies composed of hyaline substance which does not stain by Gram's method (Fig. 102). These are the

"clubs" previously mentioned, and they may occupy more or less of the circumference of the granule. In certain instances a Gram-staining filament may be seen in the central portion of a club. The main mass of this form of granule is not essentially different from that of the first-mentioned form. The characteristics of both forms of granule may be found in some granules.

The club-shaped bodies are to be regarded as products of degeneration of the marginal filaments.

In some cases isolated or small groups of filaments may be found scattered among the pus-cells in the lesions.

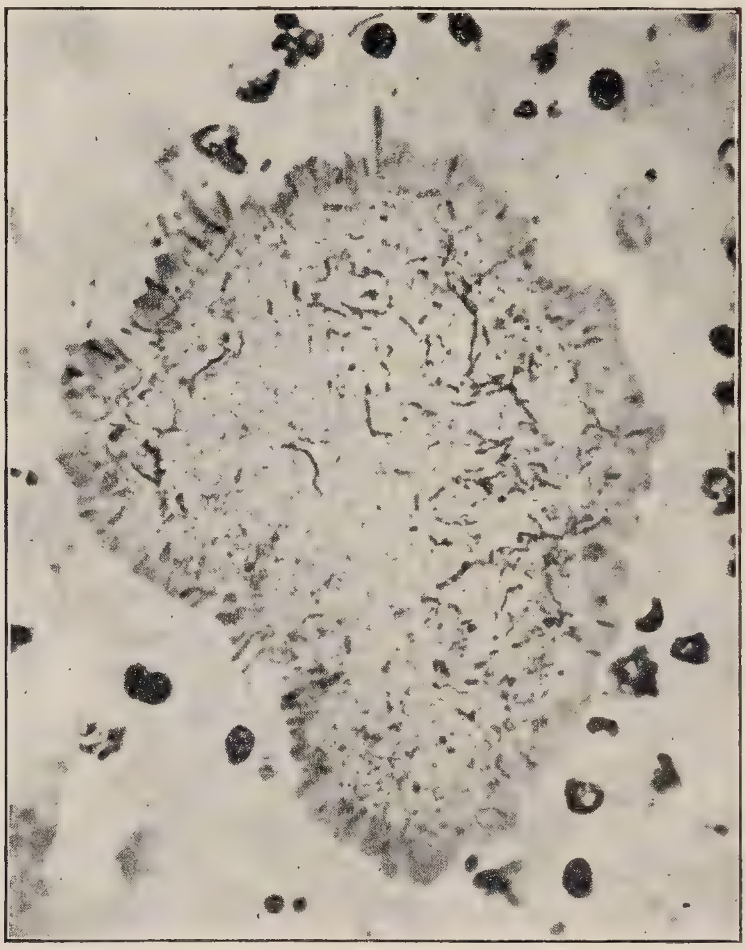


FIG. 102.—Colony or granule of actinomyces in a section through a lesion, showing the peripheral arrangement of the "clubs." In several instances the central stained filaments in the "clubs" are seen; × 750 (Wright and Brown).

Diagnosis.—The finding of the granules in suspected pus may be facilitated by spreading the pus on a slide.

The identification of the organism is made certain only when the granules have been found to present the appearances described above after crushing under a cover-glass, and after cover-glass preparations made from them and stained by Gram's method show the branching filaments.

Cultures.—Actinomyces bovis is essentially an anaërobe and it does not grow at room-temperature. A good growth in cultures is obtained only in the depths of solid culture-media and in bouillon. Growth is obtained on the surface of solid culture-media only when large numbers of the micro-organisms are planted upon the culture-media. These surface growths are white, elevated, more or less nodular, and have irregular margins.

Sugar Agar.—In "stab" cultures and in cultures by the method of 'Liborius' (see page 124), growth occurs only below a depth of about 1 cm. from the surface. The colonies continue to develop during some days in the incubator. The larger colonies are spherical, whitish, and may attain a diameter of 1 mm. or more. The smaller colonies, under the microscope, are seen to consist of a dense, interlacing felt-work of frequently branching filaments, which at the periphery are disposed in a more or less radiating manner. The microscopical colonies may be conveniently studied in thin slices cut out of the agar or in frozen sections of the agar fixed in formalin and stained by the Gram-Weigert method.

Bouillon.—Growth occurs in the form of solid, whitish masses in the bottom of the tube; there is never growth on the surface. When first isolated from the lesions the growth usually appears in the form of small, nodular, irregular, spherical, whitish structures, often adherent to one another, and forming mulberry-like masses, but under continued cultivation most of the strains of the micro-organism finally grow in the form of flaky, friable, amorphous masses, which in some instances, after some days in the incubator, become transformed into a stringy, viscid material. With most strains of the micro-organisms the bouillon remains clear. There is a good growth in bouillon, without any anaërobic precautions, apparently because the dense masses in which the micro-organism grows furnish sufficient anaërobic conditions within themselves.

Potato.—No growth.

The production of "clubs" outside of the body may be obtained by placing some of the nodular growth from a

bouillon-culture in sterile serum or pleuritic fluid and keeping it in the incubator for a few days. The filaments of the micro-organism in immediate contact with the fluid become invested with the hyaline eosin-staining sheath, and the filament thus enclosed may no longer stain by the Gram-Weigert method. In this way structures are produced which are identical in every respect with the "clubs" developed from the filaments in the lesions. (See Figs. 99, 102, 103, 105.)

Pathogenesis.—Intraperitoneal inoculation of guinea-pigs with suspensions of the growth in bouillon-cultures produces, after three or more weeks, with some strains of the micro-

FIG. 103.—Showing "club" formation about the filaments of actinomyces bovis, after exposure to the action of serous fluid outside of the animal body.

organism, granulomatous nodules in the abdominal cavity, varying in size up to 1 cm. in diameter. These nodules consist of granulation and connective tissue, enclosing small abscesses in which are found the characteristic "club-bearing" colonies or granules. Different strains of the micro-organism vary in virulence and some produce no lesions.

Method of Isolation.—The granules, preferably obtained from closed lesions, are first thoroughly washed in sterile water or bouillon and then crushed and disintegrated between two sterile glass slides. It is well to examine micro-

scopically the disintegrated material to see if filamentous masses are present, because in some instances, through degenerative changes, the filaments which represent the living elements of the granules have died out or disappeared from the granules. If no filaments are present, or if they are few in number, it is not advisable to proceed further. If, however, filaments and filamentous masses are found, then the disintegrated products of the granules are to be transferred by means of the platinum loop to melted 1 per cent. dextrose-agar, contained in test-tubes filled to a depth of about 7 or 8 cm., which have been cooled to about 40° C. The material is to be thoroughly distributed throughout the

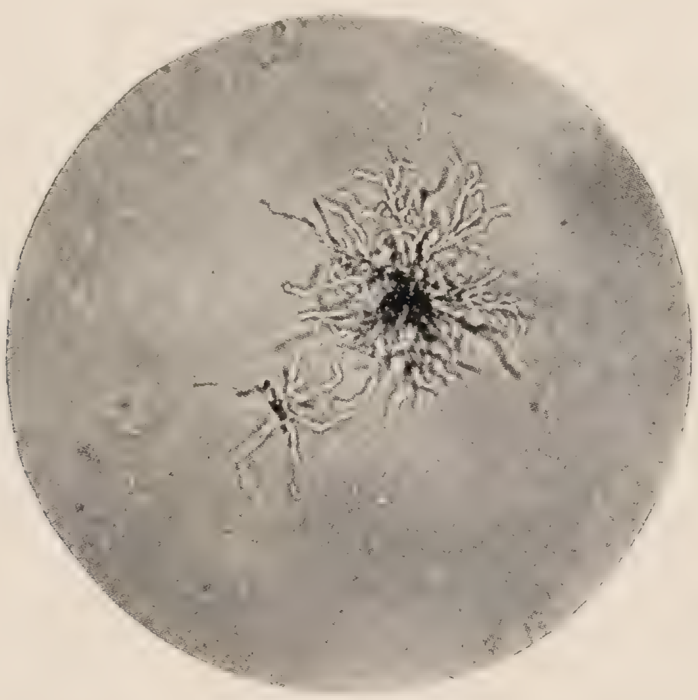


FIG. 104.—Small colonies of actinomyces bovis in the depths of an agar culture.

melted agar by means of the loop, and the tube then placed in the incubator. At the same time a number of granules, after thorough washing in sterile water or bouillon, should be placed in sterile test-tubes, plugged with cotton, and kept at room temperature in the dark.

The sugar-agar tubes, inoculated as above described, should be examined from day to day for the presence of the characteristic colonies in the depths of the agar. If very many colonies of contaminating bacteria develop in the tubes, it will probably be very difficult or impossible to isolate the specific micro-organism. If there are a few or no contaminating colonies, then the colonies of the specific micro-organ-

ism should be expected to develop in the course of two or three days to a week. If a good number of living filaments of the micro-organism have been distributed throughout the agar, the specific colonies that develop will be very numerous in the depths of the agar, especially throughout a shallow zone situated about 1 cm. below the surface of the agar-agar.

When the presence of the characteristic colonies has been determined, slices or pieces of the agar, containing colonies, are to be cut out of the tube by means of a stiff platinum wire with a flattened and bent extremity. A piece of the agar is to be placed on a clean slide and covered with a clean cover-glass. It is to be examined under a low power of the microscope, and an isolated colony selected for trans-

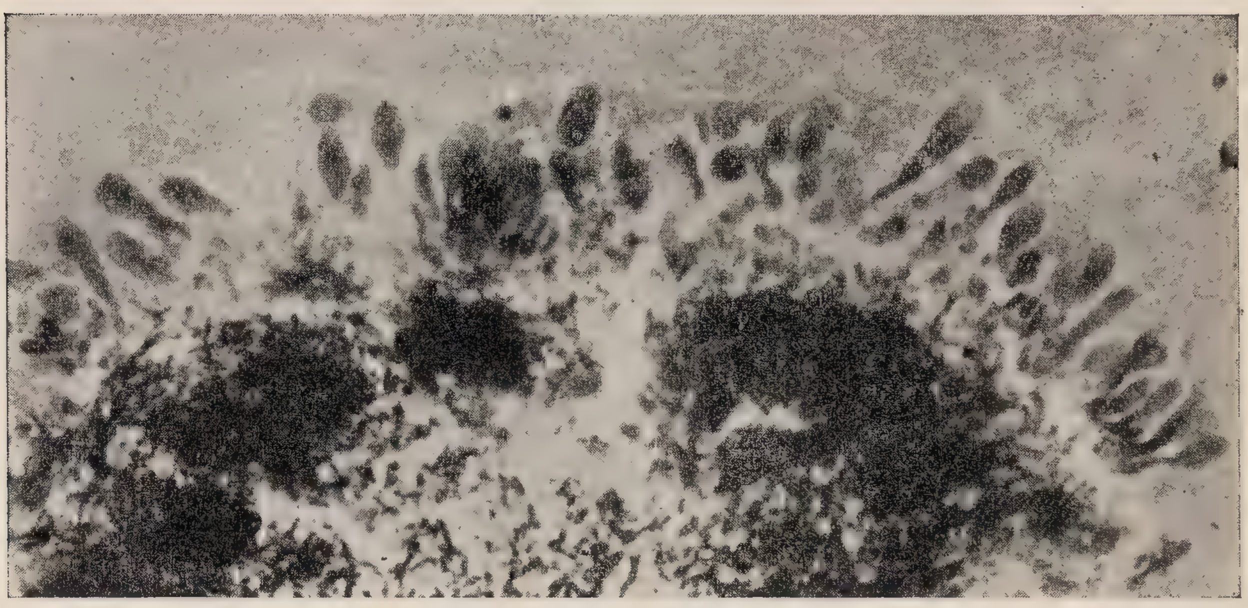


FIG. 105.—Portion of a colony of actinomyces bovis in a section from a lesion in a guinea-pig produced by intraperitoneal inoculation. The radiating "clubs" at the periphery, some with central filaments, are shown, as well as the felt-work of interlacing branching filaments in the central portions.

plantation. By obvious manipulations, under continuous control of microscopic observation, the selected colony, together with a small amount of the surrounding agar, is to be cut out, care being taken that no other colony is present. The small piece of agar thus cut out should not have a greatest dimension of more than 2 mm. The piece of agar is then transferred from the slide by means of a platinum loop to a tube of sterile bouillon, where it is thoroughly shaken up in order to free it from any adherent bacteria. If there be reason to believe that the small piece of agar has

been very much contaminated with bacteria, it should be washed in a second tube of bouillon, then the piece of agar is to be transferred by means of the platinum loop to a tube of melted sugar-agar cooled to 40° C. It should be deeply immersed in the agar and the tube placed in the incubator. If the colony thus transferred to the agar-agar is capable of growth, in the course of some days it will have formed a good-sized colony from which transplants in various culture-media may be made.

In the manner described several small pieces of agar containing single isolated colonies should be placed in sugar-agar tubes, because the chances are that some of the colonies will not grow, and contaminations with other bacteria may occur.

If the number of contaminating colonies is so great in the original agar-cultures from the granules that it is found impossible or very difficult to obtain specific colonies free from other micro-organisms, then it is probably not worth while to expend much labor with the task of isolation from these original agar-tubes, but it is much better to wait until the granules placed on the sides of sterile test-tubes have dried thereon for two or three weeks, and then proceed with these granules as just described for the fresh granules. The drying of the granules for this length of time will probably suffice to kill off most of the contaminating bacteria and enable isolated colonies of the specific micro-organism to be obtained in the agar suspension-cultures.

The Blastomycetes.—Under this heading are included those pathogenic fungi which are regarded as the infectious agents of the granulomatous and suppurative processes, known under the names of blastomycosis, oïdiomycosis, blastomycetic dermatitis, coccidioidal granuloma, and certain others.

Morphology.—In the lesions the micro-organisms appear generally as spherical bodies, each consisting of a protoplasmic mass enclosed in a double-contoured hyaline capsule. The diameter of the bodies varies up to 30 microns or more. In the protoplasm vacuoles, granules, and various

markings may be seen, but no nucleus is apparent. The mode of proliferation in the lesions in the majority of cases is by gemmation or budding. The micro-organisms in some cases have great resemblance to yeast fungi or saccharomyces. In the minority of the known cases proliferation of the micro-organisms in the lesions is not by budding, but by a process which is regarded as one of sporulation, the protoplasm of the larger forms segmenting into many small spherical bodies. Each of these small spherical bodies acquires a capsule and, being set free by the rupture of the

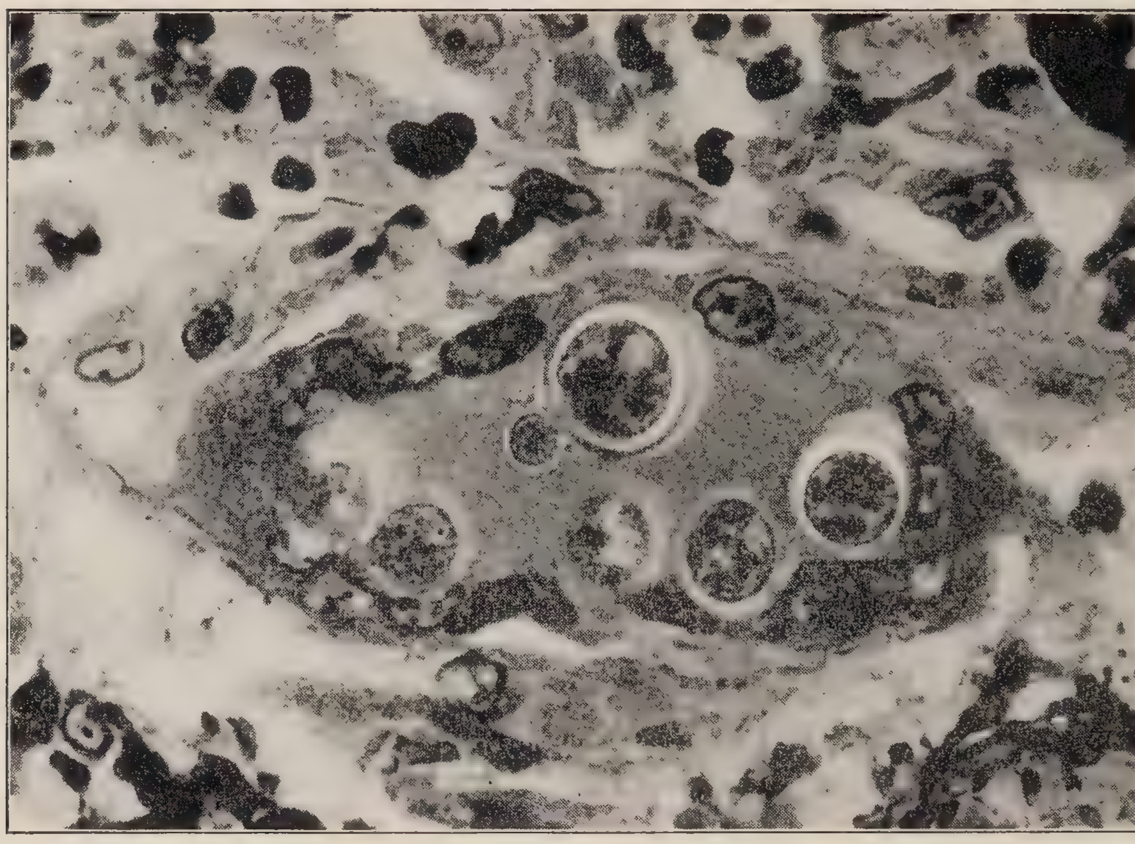


FIG. 106.—Blastomycetes in a giant-cell in the lung. Two of the organisms are budding; × about 1000 (Mallory).

capsule of the mother-cell, develops into an adult parasite. In the case reported by E. B. Wolbach, the hyaline capsule of the micro-organism was provided with prickles and spines.

Cultural Peculiarities.—The micro-organisms grow well at room temperature and in the incubator and upon any of the usual culture-media. According to H. T. Ricketts, the micro-organisms obtained from various cases may be divided into three groups, according to their biological characters, as shown in the cultures as follows:

Group 1.—Those growing chiefly as spherical or oval budding cells and resembling the yeasts, but capable of producing mycelium. The colonies on solid media are elevated, soft, moist, and white, coalescing to form a fleshy growth of paste-like consistence. Microscopically, the colonies are finely granular. In fluid media, growth appears as a flocculent sediment in a clear supernatant fluid.

Group 2.—Those forming a submerged mycelium which breaks up into chains of spores, while proliferation by budding is not a prominent feature. The colonies at first are granular and slightly elevated. Later they coalesce, become more elevated, and incorporate themselves in the medium. Eventually they form a surface like that of a piece of crumpled cloth. Microscopically, the colonies appear as masses of radiating, segmented, and branching hyphæ. In fluid media growth appears as a membrane at the surface with coherent masses or tufts at the bottom and sides of the tube.

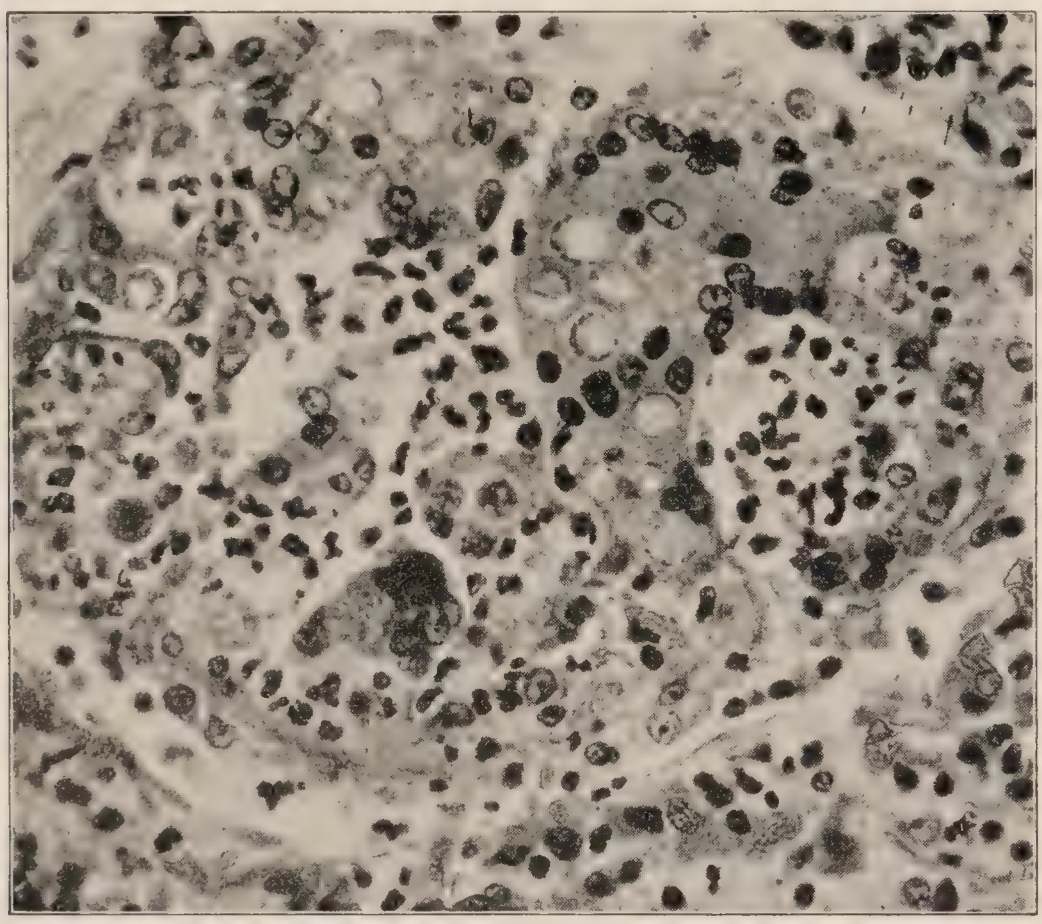


FIG. 107.—Blastomycetes in an alveolus of the lung and the inflammatory reaction caused by them; × about 250 (Mallory).

Group 3.—Those producing mycelium with fruit-bearing aërial hyphæ, and also capable of multiplying by gemmation

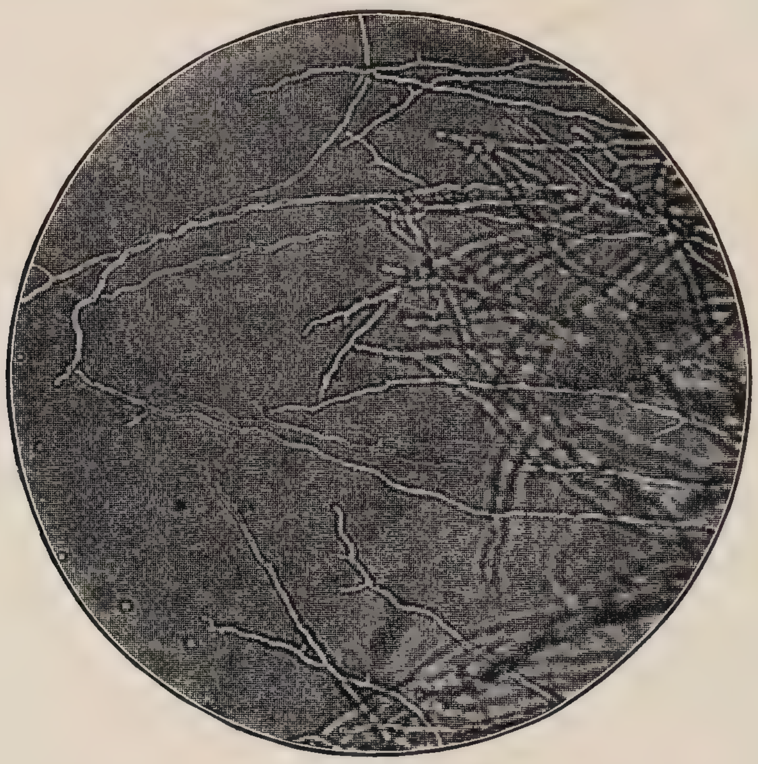


FIG. 108.—Organism of "Dermatitis Coccidioides." Edge of seventy-two-hour agar colony; × 260 (S. B. Wolbach).

FIG. 109.—Organism of "Dermatitis Coccidioides." Sprouting sphere at end of twenty-four hours' incubation, showing the development of filaments from it; × 400 (S. B. Wolbach).

or budding. The colonies are dry, white, and feathery in appearance, develop hyphæ, which eventually cover the inner surface of the tube. There is growth into the media. Micro-

scopically, the growth is made up of branching, closely segmented hyphæ with many lateral off-shoots. In fluid media the growth appears as a coherent mycelial tuft at the bottom of the tube with no surface growth.

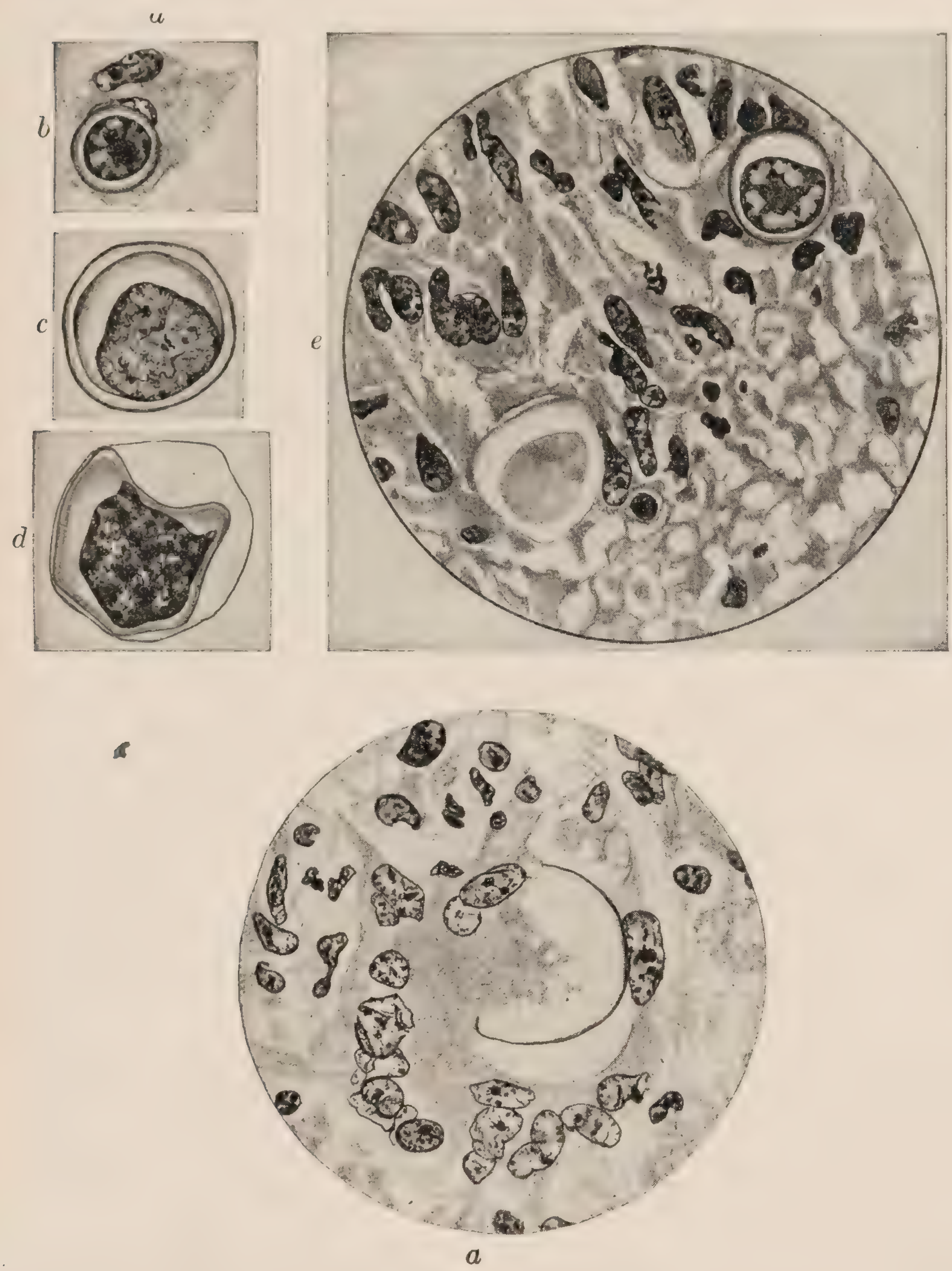


FIG. 110.—Organism of "Dermatitis Coccidioides." Various stages in the development of the organism as seen in tissue (Mallory).

The micro-organisms of the first and second groups are capable of producing fermentation, while those of the third group are not.

Pathogenesis.—In animals inoculated with cultures of some, but not all, of these micro-organisms, abscesses, granulomatous tumors, and tubercle-like nodules, widely disseminated, have been produced. In these lesions in the animals the micro-organisms exist in the same form as in the human lesions. Wolbach, working with the micro-organism of the type which proliferates in the tissues by sporulation, and which was not observed to proliferate by budding in cultures, has traced in animals the transformation of the hyphæ of the cultures into the characteristic spherical bodies of the lesions. He found that the spherical bodies arise by the segments of the hyphæ, enlarging and assuming spherical shape, the wall of the segment thus becoming the capsule of the spherical body. He also observed pointed and club-shaped hyaline bodies radiately arranged at the periphery and continuous with the capsules of the micro-organisms.

Diagnosis.—The micro-organisms may be easily recognized by mixing a drop of the pus or granulation tissue from a lesion with a little 10 per cent. solution of sodium hydrate and examining the mixture under a coverglass with the microscope.

Sporotrichum Schenckii.—This is a fungus or hypomycete which is believed to be the infectious agent in sporotrichosis, a disease characterized by the formation of gumma-like nodes, abscesses, and ulcers chiefly involving the skin and subcutaneous tissue.

Morphology.—In the lesions of the spontaneous disease the fungus elements are difficult or impossible to distinguish from cellular detritus, apparently because they exist therein in certain small spore-like forms.

In culture the fungus appears in the form of a mycelium composed of branching septate filaments with abundant formation of spores. The filaments vary somewhat in thickness, their average being about 2 microns, have refringent walls, refractive granules in their interior, and transverse septa at fairly regular intervals. The spores grow singly along the sides of the filaments and in clusters of from 3 to 6 or more at the ends of filaments, which may be slightly

expanded. They are ovate or spiculate bodies, 3 to 5 microns in their longest diameter, having a granular interior and a double contour. They are attached to the filaments by delicate pedicles, which are easily broken. With basic aniline dyes and with Gram's method of staining they stain generally evenly, but sometimes show vacuole-like areas. The filaments do not stain as deeply as the spores and may show in their interior more deeply staining granules.

Cultures.—The fungus grows on all of the usual culture-media, best in those containing sugar. It is aërobic, and

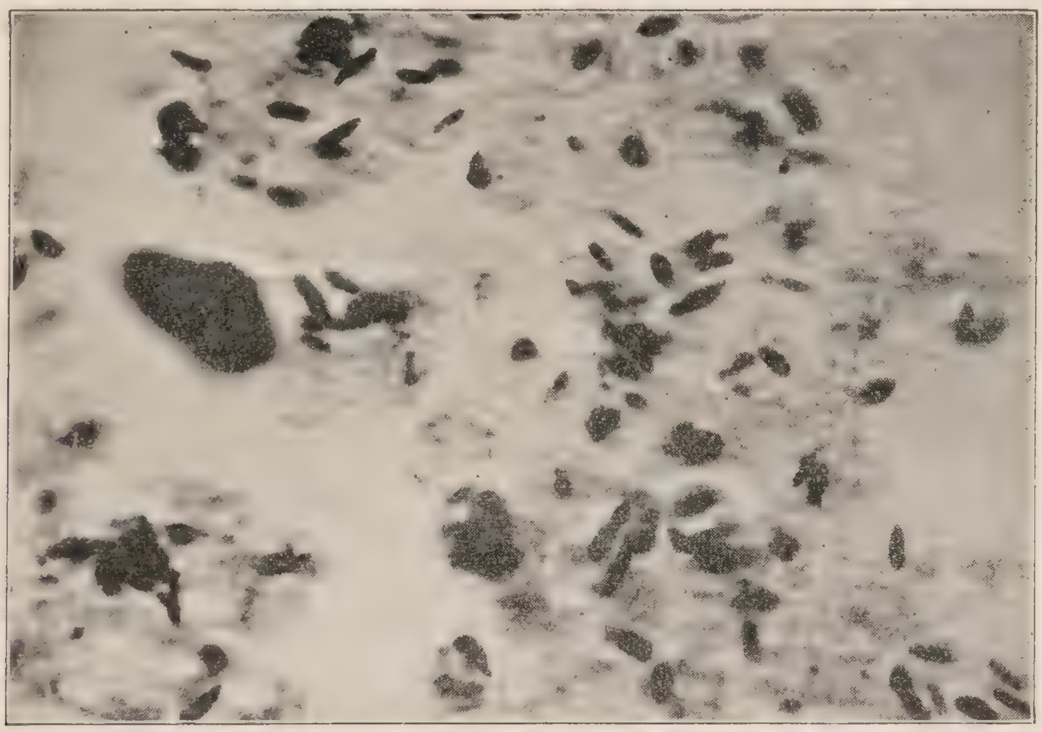


FIG. 111.—Sporotrichum Schenckii. Section of nodule in abdominal wall of white rat. Gram's stain. Cells and spores, the latter oblong and deeply colored; × about 1000 (L. Hektoen and C. F. Perkins).

thrives as well at room temperature as in the incubator. The colonies on solid media are at first pale gray or nearly white, and have a delicate fringe of radiating filaments at their margins. Later, they spread widely over the surface of the media and form a layer or a membrane 1 or more mm. thick with wrinkled surface, which may become brown or black in color and of a velvety appearance. Down-growth may occur into the underlying media. In stab cultures, lateral outgrowths occur along the line of inoculation. Gelatine is slowly liquefied by the micro-organism. In bouillon, growth appears as a downy sediment. The growth in the

original cultures from the lesions may be slow, the colonies appearing after a week or more. In succeeding generations the growth is more rapid.

Pathogenesis.—The micro-organism is pathogenic for experimental animals, especially for white rats. In the latter animals, septicemia and disseminated suppurative or granulomatous lesions may be produced by inoculation with cultures. The testicles are very frequently involved after intraperitoneal inoculation. In the experimental lesions the

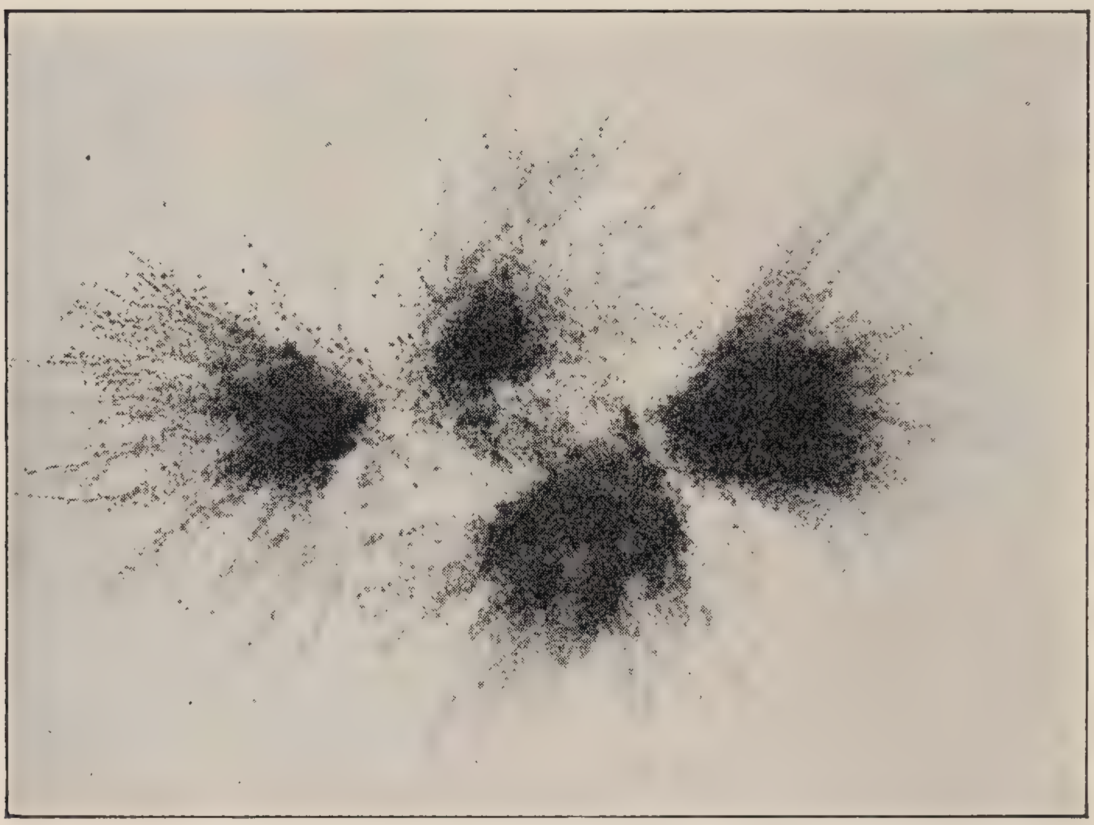


FIG. 112.—Sporotrichum Schenckii. Colonies on glycerin-agar plate. Low power (L. Hektoen and C. F. Perkins).

organism does not appear in the filamentous form, but only in modified forms, more or less resembling the spores and as oblong Gram-staining bodies, 1 to 3 microns wide and 10 to 12 microns long. Sometimes small bud-like processes project from these forms.

Occurrence.—The fungus is thought to have a natural habitat in the outer world and to be widely distributed. It is claimed that spontaneous infection with it has been observed in the dog and rat, and that some of the cases of epizoötic or mycotic lymphangitis in horses are due to infection with it.

Diagnosis.—The only practical way by which Sporotrichum Schenckii may be recognized in suspected sporo-

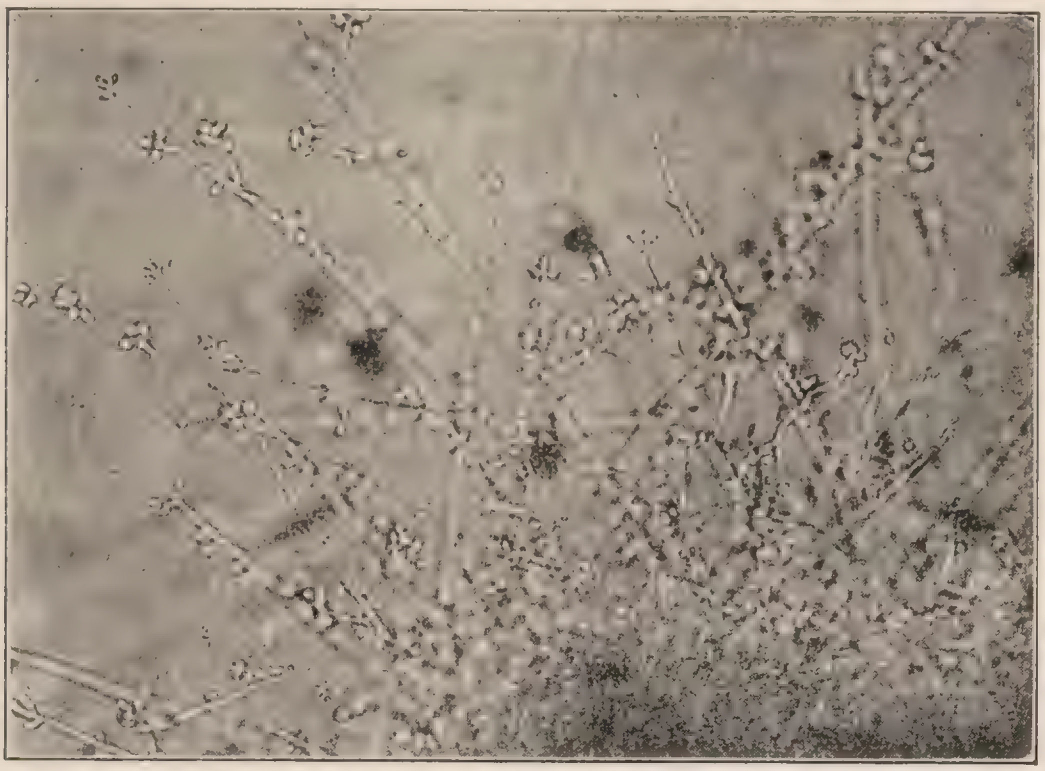


FIG. 113.—Sporotrichum Schenckii. Margin of hanging-drop culture; × about 150 (L. Hektoen and C. F. Perkins).

FIG. 114.—Sporotrichum Schenckii. Margin of hanging-drop culture; × about 1000 (L. Hektoen and C. F. Perkins).

trichosis is by obtaining it in cultures from unopened gummata or abscesses. As has been pointed out above, it is practically impossible to recognize it in lesions by direct examination.

PART III.

HISTOLOGICAL METHODS.

Introduction.—The ideal function of the technique of pathological histology is so to fix tissues for microscopic examination that every tissue-element or pathological product is perfectly preserved with all its morphological and chemical properties intact, and so to stain tissues that every tissue-element or pathological product can be readily differentiated from any other tissue-element or pathological product that resembles it. In certain respects only has this ideal yet been reached, but the number of differential stains is increasing yearly.

In the following pages the various steps in the preparation and staining of tissues have been arranged, so far as possible, in logical sequence.

LABORATORY OUTFIT.

Microscopes.—The most important laboratory instrument is the microscope. It should be, so far as means will permit, the best that skill can produce. Excellent microscopes are manufactured both abroad and in this country, but no make of microscope can be unconditionally recommended. Undoubtedly the best microscopes in every particular and the most expensive are those manufactured by Zeiss.

It is important for a beginner in microscopy, before buying a microscope of any make, to have it carefully examined and its lenses tested at a pathological or other laboratory by some one skilled in its use. The continental form of stand of medium size is to be preferred to all others. The large stand is undesirable, because it is too heavy and too high for

comfortable use. It should be furnished with rack and pinion, and with micrometer screw for coarse and fine adjustment, with a triple nose-piece, and with an Abbé condenser and iris diaphragm. The necessary objectives are a low and a high dry, and a $\frac{1}{12}$ oil-immersion. Two eye-pieces, a low and a high, will be found sufficient for all ordinary purposes.

The stands, oculars, and objectives of the Zeiss make generally used are the following:

Stands, III and IV.

Oculars, 2 and 4.

Objectives, AA, D, and $\frac{1}{12}$ oil-immersion.

Or in the apochromatic series,

Oculars, 4, 6, and 8.

Objectives, 16.0, 8.0, 4.0, and oil-immersion 2.0 mm., apert. 1.30.

Even if all these different parts cannot be purchased at the same time, it is important to buy a stand to which they afterward may be added, for the list includes only what every medical practitioner should have at his service for the proper examination of urine, sputum, blood, etc.

The apochromatic lenses and compensation oculars are too expensive to come into general use. Fortunately, they are more important for photomicrography than for general microscopic work.

The *oil-immersion lens* should always be cleaned after using by wiping off the oil with an old linen or silk handkerchief or with the fine lens-paper now manufactured for that purpose. If the lens is sticky, moisten the cloth with benzol or xylol. The same process can be used if necessary for the dry lenses, but it must be done quickly, so as not to soften the balsam in which the lenses are imbedded. Ordinarily a dry cloth is sufficient.

In using the *Abbé illuminating apparatus* it is important to bear in mind that the best results are obtained, according to Zeiss, by employing the plain mirror, for the condenser is designed for parallel rays of light. The concave mirror is to be used only when some near object, such as the window-

frame, is reflected into the field of vision or when artificial light is employed.

A *mechanical stage* is now made which can be instantly attached to any microscope. It is exceedingly useful for blood-counting or for searching carefully the whole surface of a stained cover-slip. For ordinary work it is undesirable.

For microscopic work the best *illumination* is that obtained from a white cloud, although for some purposes the light which filters through a white curtain on which the sun is shining is very useful, especially with the highest powers of the microscope. When artificial light is necessary, the Welsbach burner, or, better still, a Tungsten electric light with ground-glass globe, will be found very satisfactory. The slight yellowish tint of the light can be corrected, if necessary, by means of a piece of blue glass inserted over the mirror or just below the object to be examined.

For drawing, the *Abbé camera lucida* will be found extremely useful and convenient.

Freezing Microtome.—Freezing by means of the evaporation of ether, more rarely of rhigolene, is the method in general use. The process is both expensive and slow. A much cheaper and more rapid method of freezing was originated many years ago in the Sears Pathological Laboratory by Dr. S. J. Mixter, and has since been in constant use both here and in the hospitals in Boston. This method consists in the employment of compressed carbon-dioxid, which is found in commerce in iron cylinders containing each about twenty pounds of liquefied gas. It is commonly used for charging beer and soda-water. As a rule, the cylinders are loaned, so that it is necessary to pay for the contents only.

The cylinder must be securely fastened in an upright position near the microtome, with its valve end below and with its escape-tube on a level with the entrance-tube into the freezing-box. The cap covering the escape-tube of the cylinder should have a small hole bored through it, and into this hole a small brass tube about 5 cm. long, with a fine bore, should be tightly driven. This permits the use of a

smaller stream of gas than the escape-pipe of the cylinder would otherwise furnish. The same cap can be kept to use on all future cylinders.

The cylinder is connected with the microtome by means of a short piece of thick, strong rubber-tubing with small bore, so as to fit snugly over the escape-tube of the cylinder

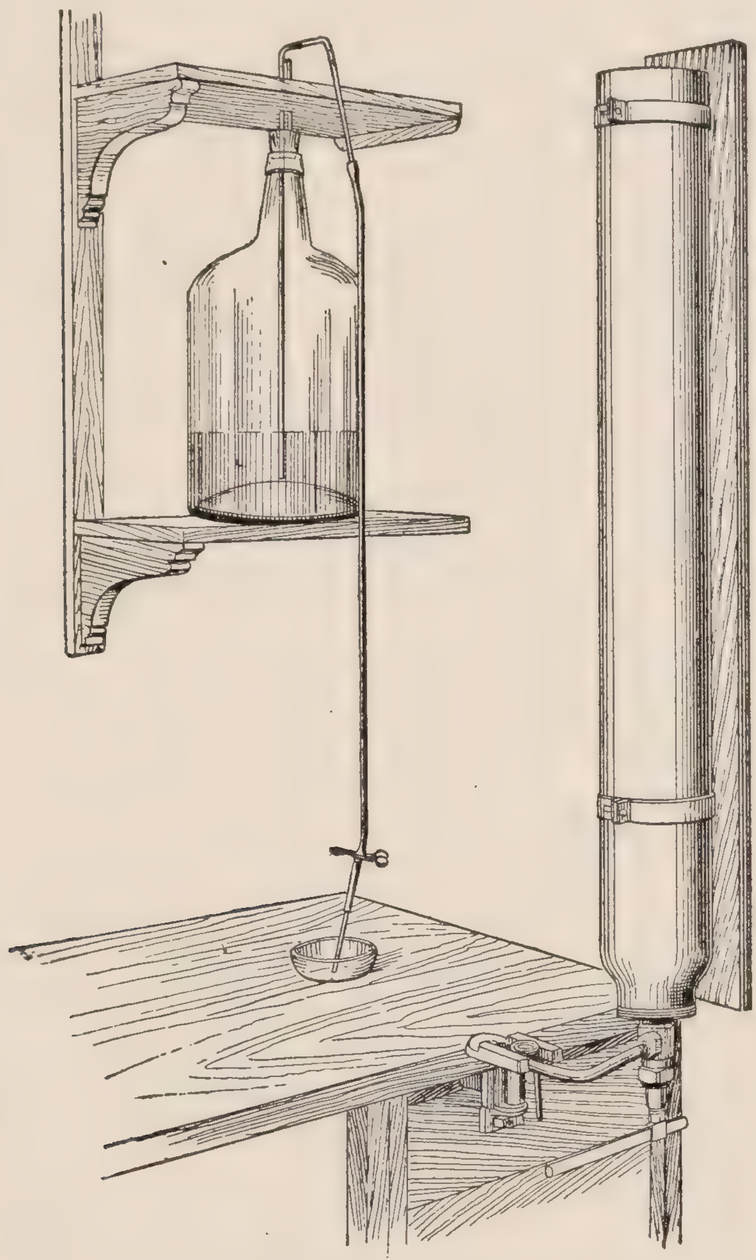


FIG. 115.—Freezing microtome.

and the entrance-tube into the freezing-box. It is advisable to wire each end of the rubber-tubing around the tube it incloses.

In order to obtain better leverage and more perfect control over the escape of the gas than are needed for the purposes for which the cylinders are ordinarily used, it is necessary to lengthen to about 25 cm., in whatever way

seems best, the handle of the key which opens the escape-valve.

The first time the cylinder is used for freezing, a little water may escape, causing considerable sputtering. In freezing, the valve should be turned carefully, so that the gas may escape slowly and evenly. Tissues fixed by alcohol or any other reagent, except formaldehyde, must be washed in running water for some hours before they can be frozen.

Even for tissue fixed in formaldehyd washing in water for ten to thirty minutes is advisable, as better sections can be obtained.

It is now possible to obtain from stores carrying automobile supplies small tubes of compressed carbon dioxid sufficient for one or two freezings. They will be found convenient for carrying to private operations when an immediate diagnosis by means of frozen sections is demanded.

The freezing-box of the microtome should be stronger than when intended for the use of ether. The Bausch and Lomb table microtome, No. 3050, with freezing attachment No. 3082, is recommended.

For cutting frozen sections the blade of a carpenter's plane, $2\frac{5}{8}$ inches wide, mounted in a wooden handle (Fig. 116), will be found very serviceable and easy to sharpen.

FIG. 116.—Knife for freezing microtome, made from the blade of a carpenter's plane.

Directions for Cutting Frozen Sections.—Sections of 10 or 15 microns in thickness or as thin as good celloidin sections, are readily obtained with most material by following these directions.

The knife must be sharp and free from nicks. It must have a chisel edge, as shown in Fig. 116. It should be sharpened by grinding on a hone, and afterward by thoroughly stropping on a razor-strop. Frequent stropping is

just as necessary as in the case of the ordinary microtome knife.

In cutting, grasp the knife by the thick wooden handle so that the end presses against the ball of the thumb and the palm of the hand, while the dorsum of the hand is uppermost; then, with the wrist flexed and held against the chest, apply the edge of the knife to the glass ways of the microtome in such a manner that the edge, bevel side downward, is at right angles to the direction of the ways and the long axis of the knife at an angle of 45 degrees to their surface; now, holding the knife and wrist rigidly in the positions just indicated, push the cutting-edge quickly forward along the ways through the specimen by moving the body forward from the waist, in the mean while pressing the cutting-edge steadily downward upon the ways with constant force. Thus a strong constant downward pressure of the edge upon the ways is maintained, and at the same time great steadiness and power are given to the cutting stroke, which are conditions that are very important for obtaining thin sections. With the fingers of the other hand manipulating the wheel of the microtome screw, a number of sections should be cut in quick succession in the manner indicated without changing the angle of the knife or the position of the hand and wrist above described, the edge of the knife on the backward movement being lifted from the ways only enough to clear the cut surface of the specimen. The sections will usually adhere to the knife, and a number of them may be allowed to collect thereon. They are removed from the knife by immersing it in water, in which they will float and flatten out, no matter how much wrinkled and compressed upon the knife they have been. The cutting of a number of sections in quick succession without pausing to remove each section from the knife seems to be necessary for obtaining the thinnest sections.

The consistence of the frozen tissue is important. The specimen immediately after freezing will usually be too hard to cut without yielding sections that break over the edge of the knife, and are, therefore, to be rejected. If this happens,

wait a few seconds and thereafter cut a section or two at short intervals until the specimen is found to have a consistence yielding satisfactory sections, whereupon a number of sections should be cut in quick succession as above described. The piece of tissue from which the sections are to be cut should not be thicker than 5 mm.

Celloidin Microtome.—There are two types of celloidin microtomes—one in which the object is raised by a screw, a second in which the object is raised by being moved up an inclined plane. The first type of machine is the better, for two reasons: the screw affords greater accuracy in the even elevation of the object than is possible with an inclined

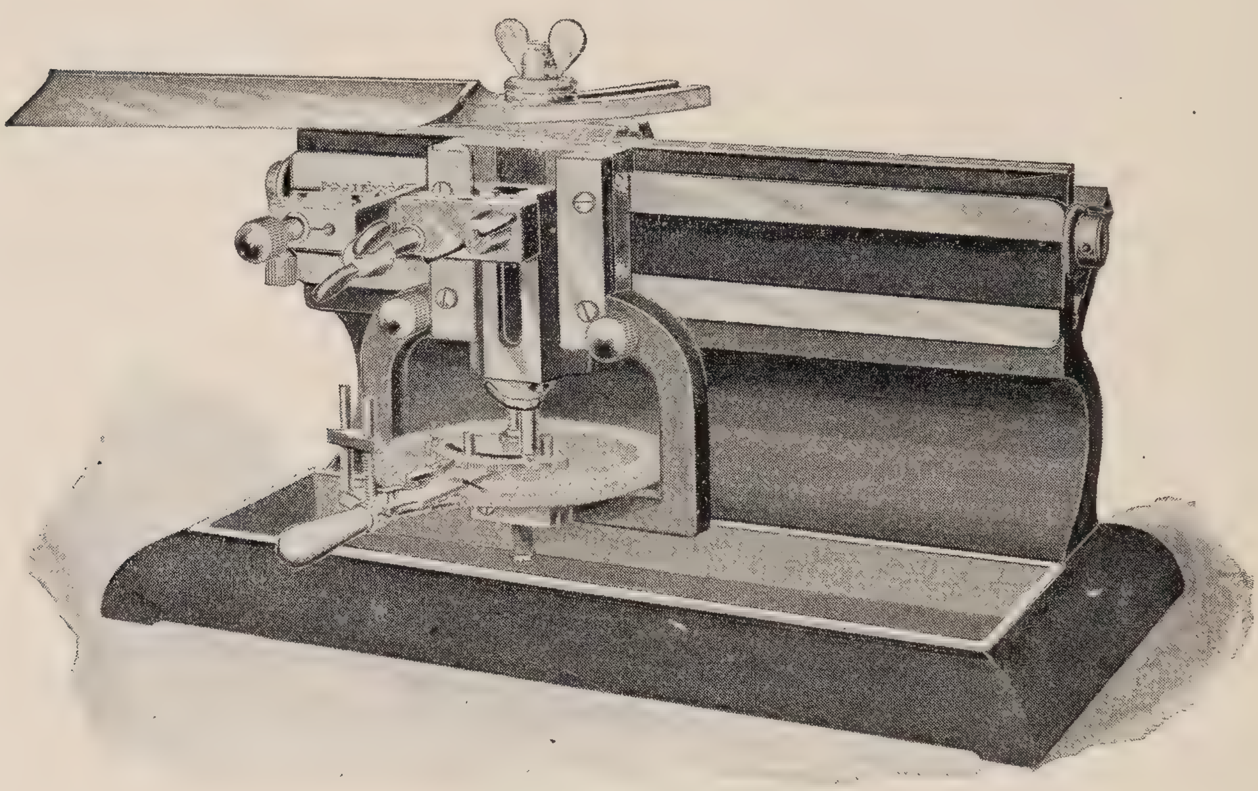


FIG. 117.—Large laboratory microtome (Bausch & Lomb).

plane, and the object remains at all times in the same relative position with regard to the knife, so that an equally long sweep of the blade can be obtained for every section. An excellent instrument of this type is made by Bausch & Lomb Fig. 117). For practical work it is much to be preferred to the elaborate Schiefferdecker-Becker microtome, designed for cutting sections under alcohol.

A new and wholly original microtome, in which the knife remains fixed and is clamped at both ends, while the object-

holder, which is raised by a screw, moves back and forth beneath the knife, has recently been designed by Dr. C. S. Minot and is manufactured by Bausch & Lomb (Fig. 119). It is intended both for celloidin and for paraffin work.

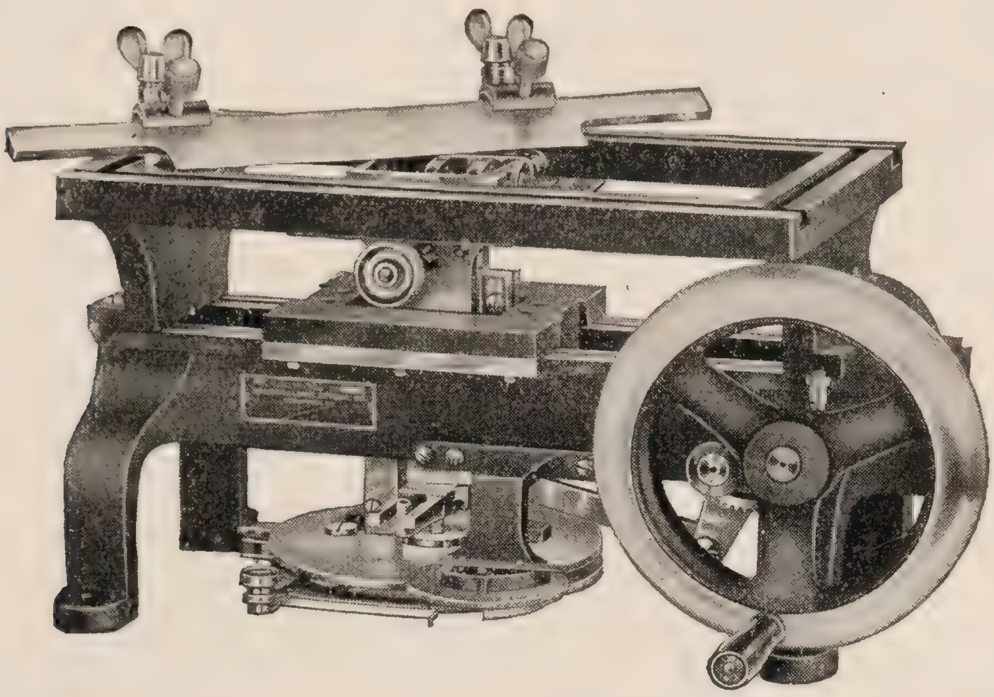


FIG. 118.—Minot precision microtome (Bausch & Lomb).

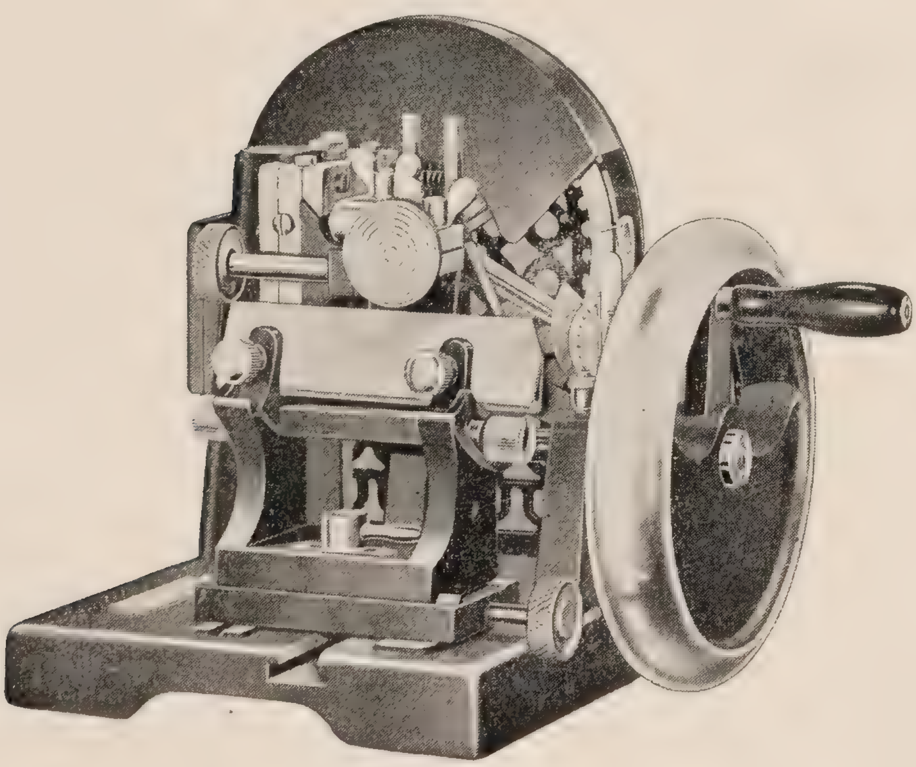


FIG. 119.—Minot's wheel microtome (Bausch & Lomb).

A *drop-bottle* on an elevated stand, with screw arrangement for regulating the amount of alcohol, is the most convenient method for keeping the object and the knife wet while cutting; 80 per cent. alcohol should be used.

Paraffin Microtome.—Although paraffin sections can be cut on a celloidin microtome, it is preferable to have an instrument designed for the purpose. Two models of the Minot wheel microtome are manufactured in this country: one by the Bausch & Lomb Optical Co., the other by the International Instrument Co., of Cambridge, Mass. The latter instrument has this advantage for pathological work: it is simple and heavy in construction, and the paraffin block-holder is controlled by a ball-and-socket joint, requiring but

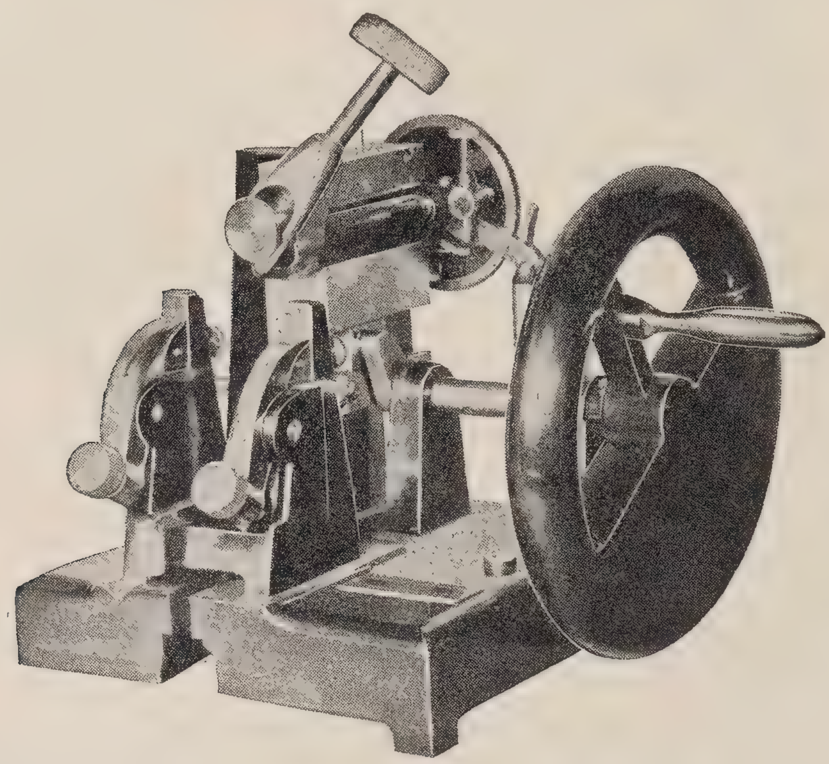


FIG. 120.—Minot rotary microtome (International Instrument Co., Cambridge, Mass.).

one screw instead of three. It has been found very satisfactory in practical use.

Paraffin Bath.—The best bath for keeping paraffin at a constant temperature is a thermostat of suitable size with hot-water jacket, such as is used for growing cultures of bacteria. The paraffin is kept in it on shelves in glass dishes of various sizes. The advantages of this method over the old way of using copper cups set into the top of a water-bath are that the paraffin is kept absolutely free from dust, each worker can have his own set of dishes, and the smallest bits of tissue can be readily found in them, because they are transparent.

Excellent paraffin, melting at 125° F. (51.6° C.), can be obtained, when bought in large quantities, for about eight cents a pound, from the regular dealers in paraffin, and can be used at all seasons of the year. (E. F. King & Co., Boston, can be recommended.)

A preliminary bath of soft paraffin is wholly unnecessary, and only prolongs the objectionable stage of heating. The regulator should register only one or two degrees above the melting-point of the paraffin.

Paraffin should be melted and decanted or filtered before use, as it often contains foreign material. When hot, it runs

FIG. 121.—Centrifuge, size 1, type B, with 8-tube, 50 c.c. head manufactured by the International Instrument Co.

through an ordinary filter without trouble. A hot-water jacket to the funnel is not at all necessary.

Centrifuge.—This instrument is of great use in obtaining quickly the sediment from various fluids, including blood and urine, and also for sedimenting and washing the red blood-corpuscles used in the Wassermann and Noguchi serum tests. The electrically run instruments manufactured by the International Instrument Co., of Cambridge, Mass., can be

highly recommended as well made, durable, and easy running. Size 1, type B, with 8-tube, 50 c.c. head will be found very satisfactory.

Vulcanized Fiber.—For mounting celloidin preparations nothing is so poor as cork, although it has been in use for years. The chief objections to it are that it does not furnish a rigid support to the imbedded object; that, unless weighted, it floats in alcohol with the specimen downward; and that it yields a coloring material which stains both the

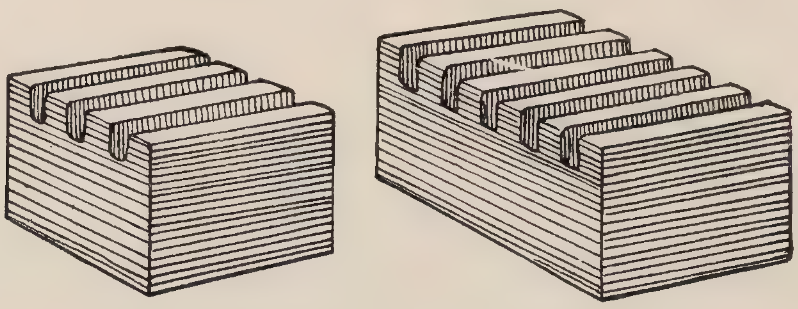


FIG. 122.—Blocks of vulcanized fiber.

alcohol and the specimen. Wood is not much better, although, of course, much firmer. Glass blocks have been proposed, and might do fairly well if there did not exist an ideal substance—viz. vulcanized fiber. This can be obtained

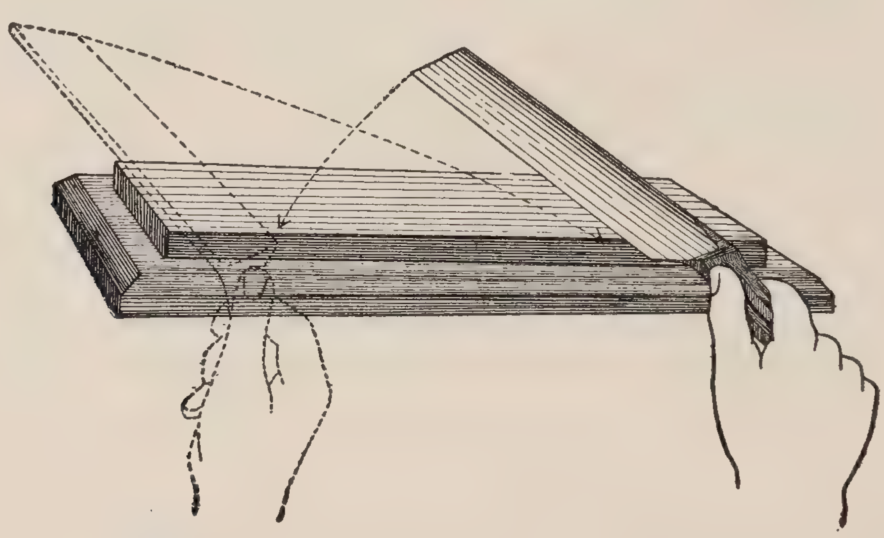


FIG. 123.—Diagram of the direction of the movements in honing.

in boards or strips, preferably $\frac{1}{2}$ or $\frac{5}{8}$ inch in thickness and sawn to any desired dimensions. It is perfectly rigid, is heavy enough to sink specimens to the bottom of the jar in an upright position, is unaffected by alcohol or water, is light red in color, so that it is easily written on with a lead pencil, gives off no coloring material, and is practically indestructible.

Several parallel cuts, 1 to 2 mm. in depth, should be sawn

into the upper surface of each block, so as to give the celloidin a firm hold.

Knives.—The knives for both the celloidin and the paraffin microtomes should be heavy and not too long, so as to afford as great rigidity as possible; they should be biconcave, so that they may be easily sharpened. It is important that every one who does much work in a pathological labor-

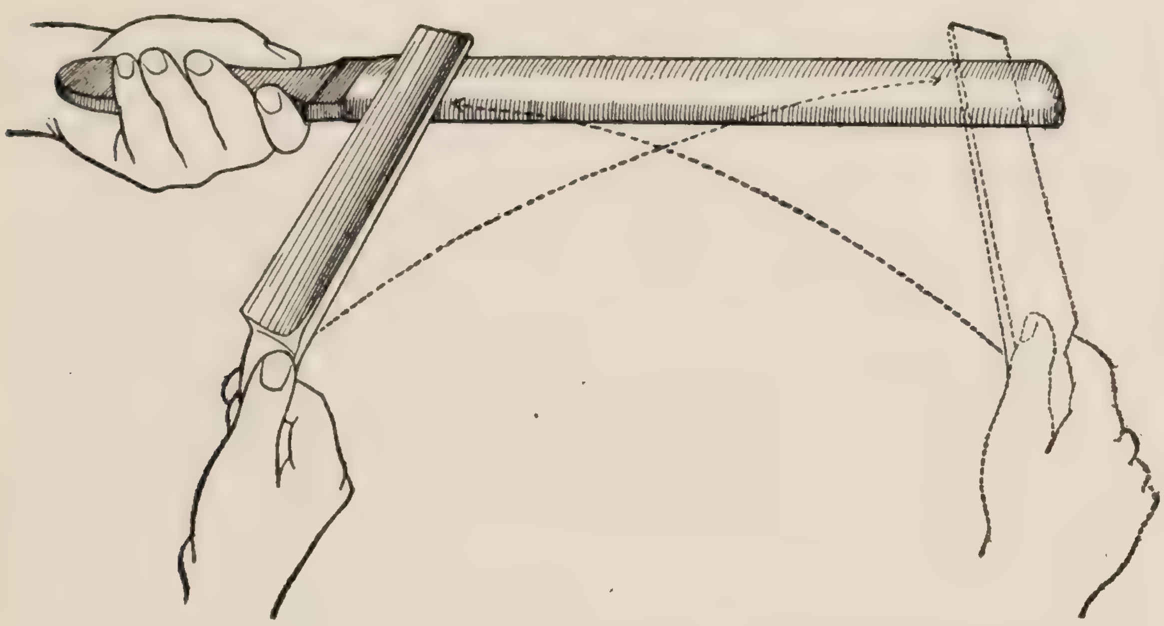


FIG. 124.—Diagram of the direction of the movements in stropping.

atory should learn to sharpen his own knives. The requisite skill is not difficult to acquire, and the time spent in learning is fully compensated for by the ability always to have a sharp knife when it is wanted. For honing a knife either a fine water-stone or a glass plate with diamantine and Vienna chalk may be used. In honing, the edge of the knife is forward and the motion is from heel to toe. The knife should always be turned on its back, and the pressure on it should be at all times rather light.

In stropping, the movement is reversed. The back of the knife necessarily precedes the edge, and the motion is from toe to heel. The direction of the movements in honing and stropping is best illustrated by the diagrams (Figs. 123, 124).

The condition of the cutting edge can be examined by drawing the knife flatwise across the low power field of the microscope. When the knife is properly sharpened the edge is smooth and even, without nicks.

A razor-strop paste greatly facilitates the smoothing of the knife edge in stropping.

Running water for washing out specimens which have been fixed in Flemming and other solutions is most easily supplied by having a water-pipe, furnished with numerous cocks 5–10 cm. apart, run horizontally over a slightly sloping shelf adjoining the sink. Attached to each cock is a rubber tube, with a glass tube in the end of it long enough to reach to the bottom of the jar (Fig. 125). By this arrangement the amount of water supplied to each specimen can be easily regulated.

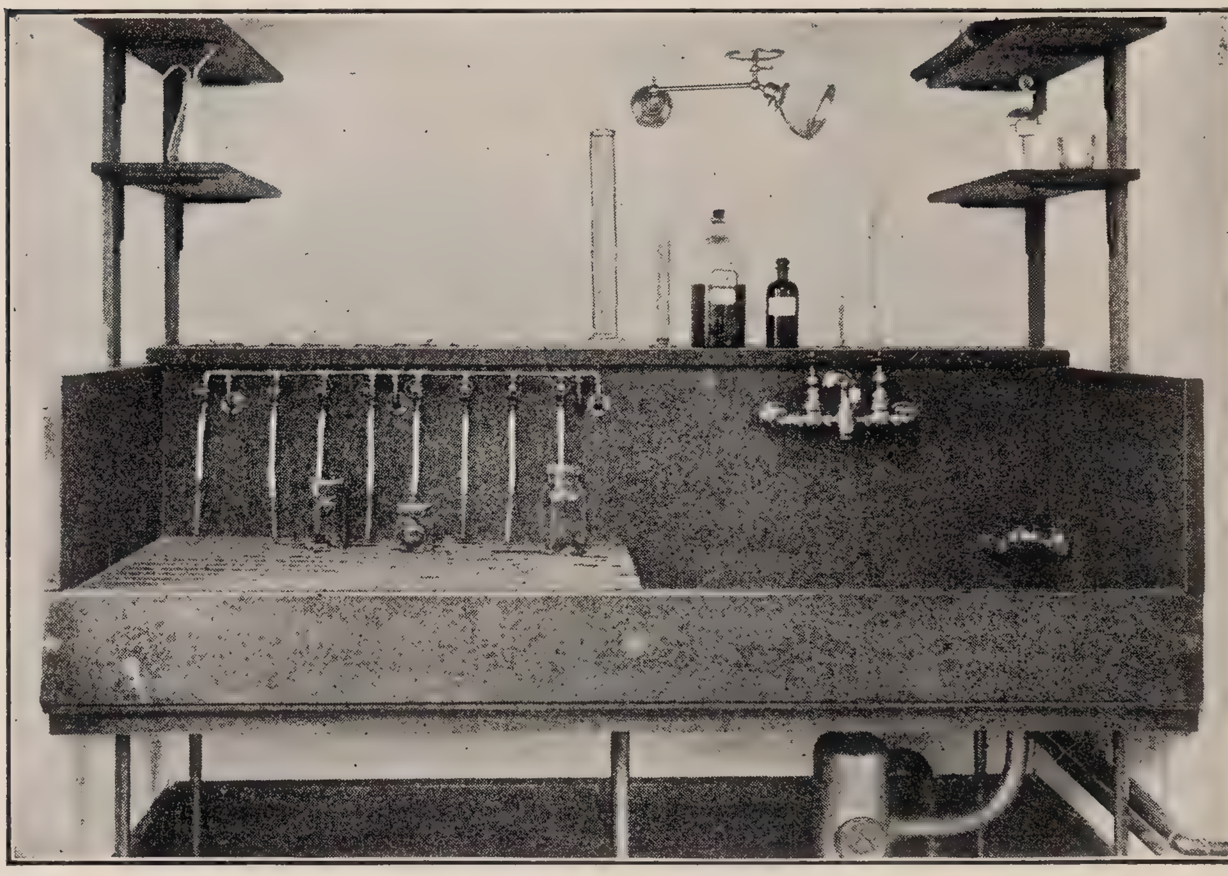


FIG. 125.—Large laboratory sink, showing adjoining shelf and arrangement for running water.

Slides should be of colorless glass with ground edges. The English form, measuring 1 × 3 inches (76 × 26 mm.), is to be preferred for ordinary use. Occasionally broader slides are needed. Thick slides are preferable to thin ones; the latter are so light that they are easily lifted by the oil-immersion lens; they also seem to warp when heated to attach paraffin sections to them. They break readily if too much pressure is applied in wiping or rubbing them.

Cover-slips should be square or oblong according to the shape of the specimen. Most dry lenses are adjusted for cover-glasses measuring 16 or 17μ in thickness, so that if

possible no cover-slip ranging outside of 15 to 18μ should be used. With an oil-immersion the exact thickness is not quite so important.

Slides and cover-slips are cleaned by dipping in alcohol and wiping dry with a soft crash towel or old linen handkerchief.

Coverslips, after they are clean, should be preserved dry in covered dishes. The common method of keeping them under alcohol cannot be recommended.

To clean old slide preparations, heat them until the balsam softens so that cover-slips and slides can be drawn apart. The slides and cover-slips are then treated separately with nitric acid. A 10 per cent. solution is usually sufficient, but occasionally the strong acid will be found necessary. Some workers prefer equal parts of alcohol and hydrochloric acid; still others the following mixture:

Bichromate of potassium,	2 parts;
Sulphuric acid,	3 "
Water,	25 "

A thorough washing in running water, followed by alcohol, completes the process. Alkalies are not so good for cleaning purposes, because they attack the glass.

Staining Dishes.—Watch-glasses are not satisfactory on account of their instability. Concave dishes with flat bottoms are much better for ordinary use, and can be obtained of several patterns. They should be large enough to hold 25 c.c. of fluid. The *Syracuse solid watch-glasses* are very good dishes of this shape. Individual glass butter dishes can be obtained which are very satisfactory and comparatively cheap.

Stender dishes of various sizes will be found useful for many purposes.

The "*New Practical Staining Dish*" (No. 16,618) manufactured by the Bausch and Lomb Optical Co. is very useful for staining at once a number of paraffin sections.

Oblong rectangular Petri dishes are very convenient for staining preparations mounted on the slide.

For staining at once a large number of sections for class

purposes, the *Hobb's Tea Infuser* has been found very useful. If set in a small lemonade cup, but little stain is required.

Large concave dishes holding 200 c.c. will be found the most convenient for holding frozen sections of fresh tissue, because a slide can be dipped into them and under the sections. They are known in the trade as glass nappies.

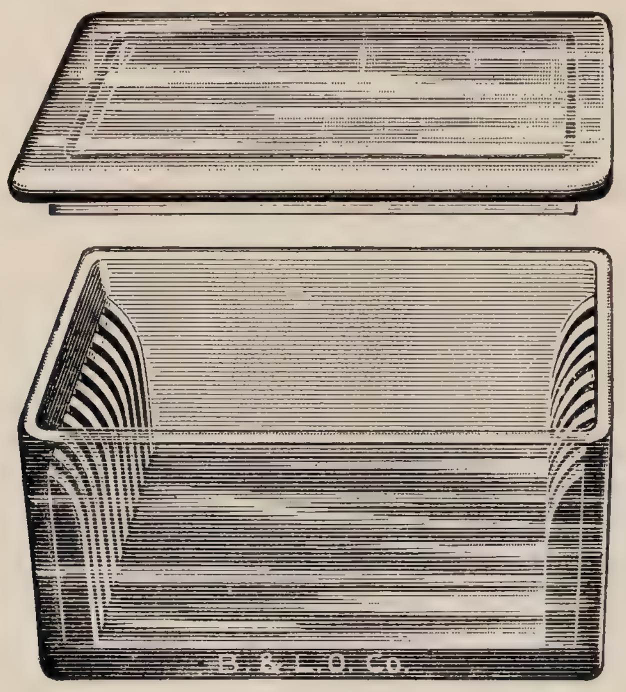


FIG. 126.—New practical staining dish.

Large flat-bottomed glass dishes known as *crystallizing dishes*, holding one to three liters, are excellent to fix tissues in, as they allow the thin slices of material to lie flat. If several sizes are obtained the larger dishes serve as good covers for the smaller ones.

Metal Instruments.—Spatulas of different sizes are

FIG. 127.—Spatula.

needed. They should be thin, smooth, and large enough, so that a section will not curl over the edge (Fig. 127).

The best instrument for transferring sections under all circumstances is a piece of platinum wire mounted in an ordinary screw needle-holder. It is pliable and can be bent to any shape, will not break like a glass needle when dropped,

and is not affected by acids. Ladies' hat-pins form a cheap but serviceable substitute. They are readily bent to any desired shape by heating. Forceps, scissors, scalpels, and many other instruments required in microscopical work do not need any special mention.

Bottles.—For cover-slip work and for staining on the slide dropping-bottles will be found extremely convenient. The patent T. K. pattern of 50 c.c. capacity, with flat top, is probably the best form and size.

For stains and reagents, glass- and cork-stoppered bottles of various sizes are required. The sizes most used are those containing 125, 250, 500, and 1000 c.c.

The lightning jars of half-pint and pint capacity, such as are employed for preserving fruit, can be highly recommended for holding pathological tissues after fixation. Wide-mouthed 100 c.c. bottles are useful for holding small amounts of tissue.

METHOD FOR BLACKENING TABLE TOPS.

Solution I.

$$\left.\begin{array}{l}\text{125 grams copper sulphate}\\ \text{125 grams potassium chlorate}\\ \text{1000 c.c. water}\end{array}\right\}\text{Boil until dissolved.}$$

Solution II.

150 grams anilin hydrochlorate,
1000 c.c. water.

Use white wood for tops, since it is cheaper and takes stain better than pine. Oak gives good results.

Spread papers to cover floor completely under and around tables.

1. Apply with brush two coats of Solution I *while hot;* the second as soon as the first is dry.

2. When second is dry, apply in the same way two coats of Solution II and let them dry thoroughly.

3. Put on with a *cloth* thin coat of raw linseed oil and polish thoroughly by rubbing.

4. When dry wash thoroughly with *hot soapsuds.*

5. Rub again with raw oil.

6. Rub with cotton waste over and over again until the black does not come off. This requires hard work.

To keep tops in perfect condition, rub off once a week with white (liquid) petrolatum.

N. B.—If table tops are blotched with paraffin, wax, or resin, remove these before beginning to paint. Stains do not harm. Paint top surface and edges only.

EXAMINATION OF FRESH MATERIAL.

Fresh tissues may be examined either in teased preparations or in sections.

Teased preparations are made by cutting out a very small bit of the tissue in question and dividing it as finely as possible, by means of two sharp, clean needles, on a slide in a drop or two of some indifferent fluid, such as the normal salt solution. Teased preparations are often made, for instance, of the heart-muscle when fatty degeneration is suspected. If the tissue is soft, the cells are easily obtained by simply scraping the cut surface with the edge of the knife.

Sections of fresh tissues can be made with a razor or with a double knife, but much the better way, at least for general diagnostic purposes, is to use frozen sections, which can be very quickly and perfectly made with the freezing microtome. The fresh sections are put into salt solution or into ordinary tap water in a glass dish large enough to permit of a slide being dipped into it, so that a section can be floated and spread out evenly on its surface. The slide is then carefully raised, the excess of fluid is wiped off, and a coverslip put on.

If it is desired to stain the section, a few drops of Löffler's methylene-blue solution, diluted 1 to 3 with water, are poured over it after it is spread evenly on the slide. Stain for twenty seconds. Then float the section off in a dish of water and wash out the excess of stain. Remount the section on the slide, wipe away the excess of water, and drop a coverslip on

the preparation. If sections of fresh tissues are put directly into a staining fluid in the ordinary manner, they pucker up and do not stain evenly.

Sections of fresh tissue may be fixed, stained, cleared, and mounted in balsam by a slight modification of the method for frozen sections given on p. 271. This modification consists in covering the section with 95 per cent. or absolute alcohol after it has been spread out evenly on the slide as described in the method referred to. The alcohol is to be dropped on the section carefully from a drop bottle, in order to avoid folding. After thirty seconds the alcohol is drained off and the section flattened out on the slide with blotting-paper and further treated according to the method above mentioned. If the section is not treated with alcohol before blotting, it will adhere to the blotting-paper and not to the slide.

Fresh preparations are often treated with chemicals for various purposes. Of these chemicals, *acetic acid* is the most generally useful in pathological work. It shrinks the nuclei and renders their outlines more distinct. It swells connective tissue, making it more transparent, so that the elastic fibers which are unaffected stand out distinctly. It precipitates mucin and dissolves or renders invisible the albuminous granules so abundantly present in the cytoplasm in the cloudy swelling of various organs in disease. Its main use as a reagent for fresh tissues is to demonstrate fat and to differentiate that substance from albuminous granules.

Acetic acid is ordinarily used in a 1 to 2 per cent. aqueous solution, a few drops of which are placed at one edge of the cover-slip, and then drawn beneath it by placing a piece of filter-paper on the opposite side. If in a hurry, however, stronger solutions, or even glacial acetic acid, may be used. Other reagents are of less importance, but are occasionally used.

Osmic acid is sometimes employed in a 1 per cent. aqueous solution to demonstrate fat, which it stains brown to black.

An alcoholic solution of *Scharlach R.* is being used more

and more for the same purpose. It stains fat orange to red.

Hydrochloric acid in a 3 to 5 per cent. solution is used to demonstrate calcification. Phosphate of lime is simply dissolved, while from carbonate of lime bubbles of carbon-dioxid (CO_2) are set free.

Indifferent Fluids.—Fresh tissues are usually examined in normal salt solution—a $\frac{6}{10}$ per cent. solution of common salt in water. It has the advantage over water that tissues do not swell up so much in it, blood-corpuscles are unaffected, and the finer structures are better preserved. A very few drops of Lugol's solution added to the stock-bottle of salt solution will be found useful in preventing the growth of mould.

Serous fluids, such as hydrocele fluid, are occasionally used. Artificial serum is made by adding 1 part of egg-albumin to 9 parts of normal salt solution.

Macerating fluids are little used in pathology. Occasionally, however, when tissues are tough, so that they cannot be readily teased apart, they are macerated in certain fluids which dissolve the substances that hold the different elements together. The reagents most commonly used are the following:

1. *Ranvier's one-third alcohol* is made by taking 1 part of 96 per cent. alcohol and 2 parts of water; twenty-four hours are usually enough.

2. Very dilute solutions of *chromic acid* are recommended —$\frac{1}{100}$ to $\frac{1}{300}$ of 1 per cent.

3. *33 per cent. Caustic Potash.*—Tissues are macerated in a few minutes to one hour: they must be examined in the same fluid, because the cells are destroyed if the solution is weakened.

Examination of Fluids.—Small fragments of tissue should be picked out with forceps. If much blood is adherent, wash the tissue well in salt solution. When the cellular elements are few in number, they are obtained with a pipette, just as in urine-work, after allowing them to settle at the bottom of the glass. A centrifugal machine will be found of great service when the sediment is slight.

INJECTIONS.

INJECTIONS are not much used in pathology. The process is an art that requires much patience and considerable experience. The purpose of an injection is to render vessels and vessel-walls more visible than under ordinary circumstances. Transparent, deeply colored fluid mixtures are used, which will become hard in the vessels. Some injection-masses are employed cold, others warm. The warm injection-masses contain gelatin, and are much more troublesome to use, but give much the more perfect results. For coloring the mass carmine is the best material, because it is a permanent color.

The instruments required are cannulas of various sizes and a syringe, or, better still, a constant-pressure apparatus.

When a warm injection-mass is used, the bottle containing the mass must be placed in a water-bath and kept at a temperature of about 45° C. The organ or animal to be injected must likewise be placed in a water-bath of the same temperature.

It is very important that in connecting the end of the tube carrying the injection-mass with the cannula inserted in the vessel no air-bubbles shall enter. When blood-vessels are to be injected, it is advisable to wash them out first with normal salt solution.

Cold Injection-masses.—1. *Blue Coloring Mass.*

Soluble Berlin blue,	1;
Distilled water,	20.

2. *Carmine Injection-mass* (Kollmann).—Dissolve 1 gram of carmine in 1 c.c. of strong ammonia plus a little water; dilute with 20 c.c. of glycerin. To this solution add 1 gram of common salt (NaCl) dissolved in 30 c.c. of glycerin. To the whole solution add an equal quantity of water.

Fischer has obtained good results by washing out the vessels in the usual way with physiological salt solution, or, better still, with a fibrin-dissolving fluid such as a freshly filtered 8 per cent. solution of nitrate or sulphate of sodium and then injecting good fresh

milk. Fix the tissues for at least twenty-four hours in a 10 per cent. solution of formalin plus 2 per cent. of acetic acid.

Cut frozen sections and stain with Scharlach R. Counterstain in alum-hematoxylin. Mount in glycerin. The fat can also be stained with osmium tetraoxid. The capillaries are outlined by the fat emulsion stained red or black.

Warm Injection-masses.—1. *Berlin Blue.*—Warm the solution of Berlin blue given above, and add it, with continual stirring, to an equal quantity of a warm, concentrated solution of gelatin prepared as follows: Allow clean sheets of the best French gelatin to swell up for one to two hours at room-temperature in double the quantity of water. Then dissolve them by warming gently over a water-bath. Filter the combined solution through flannel.

2. *Carmine-gelatin Mass.*—This is by all means the best injection-mass to use, because it is permanent, but it is very difficult to prepare.

Dissolve 2 to 2.5 grams of best carmine in about 15 c.c. of water, to which just enough ammonia is added, drop by drop, to effect the solution. Filter the fluid obtained, and add it, with continual stirring, to a filtered warm, concentrated solution of gelatin (prepared as above) over the water-bath. Then add acetic acid slowly until the color changes to a bright-red shade. The exact amount desired is when the solution loses its ammoniacal odor and has a peculiar sweetish aroma free from acid. Examined under the microscope, no granular precipitate of carmine should appear. If too much acetic acid has been added, so that the carmine is precipitated, the mass must be thrown away and a new lot prepared.

Organs which have been injected with a cold mass are placed directly in 80 per cent. alcohol. After a few hours they are to be cut up into pieces that are not too small. After a warm injection-mass the organ or animal is placed first in cold water to hasten the solidification of the gelatin, and then transferred to 80 per cent. alcohol. Masses already prepared for injecting cold or warm can be obtained from Gruebler.

FIXING REAGENTS.

The various reagents used for the preservation of fresh tissues possess the properties of penetrating, killing, fixing, hardening, and preserving in different degrees. Of these properties "fixing" is the most important, and to a certain extent implies or includes the others. The term "fixative" has been used more particularly, perhaps, for reagents which preserve faithfully the various changes of the nucleus in karyomitosis. In a broader sense, however, it refers to the faithful preservation of any tissue-element or pathological product, and of the chemical properties peculiar to that element or product. A good fixative is a reagent that penetrates and kills tissues quickly, preserves the tissue-elements, and particularly the nuclei, faithfully in the condition in which they are at the moment when the reagent acts on them, and hardens or so affects them that they will not be altered by the various after-steps of staining, clearing, and mounting. Most fixatives are mixtures of different reagents so combined that all the desirable properties may be present in as large a degree as possible.

The choice of the proper fixing reagent for a given tissue is often difficult, and must depend largely on the nature of the pathological lesions present or suspected, and on the purposes for which the tissue is preserved. The best general fixative yet devised for faithful preservation of all kinds of tissues is Zenker's fluid. It is recommended above all others after many years of constant trial. Orth's fluid, perhaps, ranks next, but does not permit nearly so great a variety of stains to be used after it. It has the advantage of costing much less. As a general fixative for all sorts of tissues when the main desire is to obtain reasonably faithful fixation for diagnostic purposes, formaldehyde has to a large extent replaced alcohol. It permits about all the chemical reactions to be performed which are possible after alcohol fixation, and has the additional advantage of preserving fat of all kinds, and especially the myelin in the sheaths of nerve-fibers.

It is strongly advised that in all important cases tissues be hardened both in Zenker's fluid and in formaldehyde: in Zenker's fluid for general histological study, and for the preservation of nuclear figures, bacteria, and fibrils of all kinds; in formaldehyde for the preservation of fat, myelin, and various substances, such as amyloid and hemosiderin, to which it may be desirable to apply chemical tests. For certain specific purposes other fixatives are sometimes required, such as alcohol for the preservation of glycogen and sodium urate crystals, and corrosive sublimate for mucus.

Tissues fixed in a solution of formaldehyde or in alcohol may remain as long as desirable in those fluids. Tissues hardened in most of the other fixatives must be transferred, after thorough washing in water, to alcohol for preservation. It is usually recommended to pass the specimens through graded alcohols, either through 30, 60, 90, and 96 per cent., or through 50, 70, and 96 per cent., allowing them to remain from a few hours to a day in each strength. For most purposes it will be found sufficient to transfer the specimens directly from water to alcohol of 70 to 80 per cent., in which they may remain until it is desired to imbed them.

Alcohol extracts chrome salts from tissues hardened in solutions of them. As these salts are precipitated in the alcohol under the action of light, it is desirable, although by no means necessary, to keep all such specimens in the dark.

Zenker's Fluid.—

Bichromate of potassium,	2.5	grams;
Corrosive sublimate,	5	grams;
Water,	ad 100	c.c.;
Glacial acetic acid,	5	c.c.

Dissolve the corrosive sublimate and the bichromate of potassium in the water with the aid of heat.

The solution is practically Müller's fluid saturated with corrosive sublimate, plus 5 per cent. of glacial acetic acid. The sulphate of sodium is omitted because it serves no useful purpose. Do not add the acetic acid to the stock solu-

tion, but only in the proper proportion to the part taken for hardening pieces of tissue, because the acid evaporates so readily, and also produces changes in the chrome salt.

Directions for Use.—1. Fix tissues in the solution twelve to twenty-four hours.

2. Wash in running water twelve to twenty-four hours.

3. Preserve in 80 per cent. alcohol until used.

Tissues float at first in this solution, which penetrates fairly quickly. The greatest drawback to the fluid is the precipitation of a mercuric oxid, which takes place to a varying degree in the tissues. This precipitation can be removed by means of iodin, which forms a colorless, soluble compound. Do not add iodin to the alcohol in which the tissues are preserved, because prolonged treatment with iodin exerts an injurious effect on the staining properties of the cells. Embed the tissues and cut sections without removing the precipitate, and then treat the sections, just before staining, with Lugol's solution or a 1 per cent. alcoholic solution of iodin for ten to twenty minutes, followed by alcohol to remove the iodin.

Zenker preparations stain slowly but beautifully in alum-hematoxylin. The most brilliant results, however, are obtained by staining with eosin, followed by Unna's alkaline methylene-blue solution. Excellent results are also obtained after staining in phosphotungstic-acid hematoxylin, and by the anilin-blue method. They bring out fibrin and various kinds of fibrils in addition to nuclear details.

Orth's Fluid.—This is a general fixative consisting of the well-known Müller's fluid plus 4 per cent. of formaldehyde:

Bichromate of potassium,	2 to 2.5;
Sulphate of sodium,	1;
Water,	100;
Formaldehyde (40 per cent. solution),	10.

The sulphate of sodium is probably best omitted. The formaldehyde should be added only at the time of using,

for in two days the solution becomes darker, and by the fourth day a crystalline deposit begins to take place. As fixation is ordinarily complete in three to four days, this deposit does not matter. The tissue should not be over 1 cm. in thickness. Small pieces, $\frac{1}{3}$ to $\frac{1}{2}$ cm. in thickness, can be readily hardened in the incubator in three hours. The specimens should be washed thoroughly in running water six to twenty-four hours before placing in 80 per cent. alcohol.

The method is particularly recommended for mitosis, red blood-corpuscles, bone, and colloid material (in cystomata, etc.), as it gives a very good consistence to the tissues, but the histological detail is not so good as after Zenker's fluid. The addition of 5 per cent. of acetic acid would unquestionably improve it.

Formaldehyde.—The gas formaldehyde (HCOH) is soluble in water to the extent of 40 per cent. Solutions of this strength are manufactured by different commercial houses under the names of formaline, formol, and formalose. The best strength of formaldehyde to use for fixing tissues is a 4 per cent. solution; that is, 10 parts of the aqueous 40 per cent. solution, no matter what name is given to it, to 90 parts of water. The addition of 5 per cent. by volume of glacial acetic acid to this solution is said to improve its fixing properties, but tissues cannot be left in the mixture. They must be transferred after twenty-four hours to the plain formaldehyde solution.

This fixing reagent penetrates very quickly. Its hardening action is not understood. It does not precipitate albuminous bodies, but makes them quite firm. It also hardens nerve-sheaths, acting toward them and red blood-corpuscles like the chrome salts. Formaldehyde is very useful for preserving gross specimens, because it gives them a rather tough elastic consistence and preserves the normal color better than other hardening fluids and also the transparency of many parts, such as the cornea. In general histological work formaldehyde is largely used now-a-days as a fixative in place of alcohol. It possesses the great advantage that

frozen sections are easily made of tissues fixed in it. In addition it serves two very useful purposes: it preserves fat and myelin so that they can be easily stained and demonstrated by special methods. Unfortunately, formic acid frequently develops in formaldehyde and exerts an injurious action on tissues preserved in it.

As a fixative for specimens that are to be embedded in paraffin it is not recommended unless combined with other reagents, such as bichromate of potassium in Orth's fluid, because it does not appear to harden the tissue elements sufficiently to enable them to resist the shrinking effects of prolonged exposure to alcohol and heat in the process of embedding. In frozen sections, however, prepared by the method described below, this shrinkage of the tissue elements is not apparent, probably because prolonged exposure to dehydrating, clearing, and embedding agents is avoided.

Wright's Method for Frozen Sections.—This method has given such satisfactory results as a histological procedure, and has proved, after an exhaustive testing in practical work, to be such a saver of time, labor, skill, and expense in obtaining satisfactory sections adequate for most routine purposes, that it seems destined to replace extensively the celloidin and paraffin methods. The essential feature of the method is the fastening of the section to the slide immediately after cutting, thus keeping even the most fragile or irregularly shaped sections intact and smooth during the processes of staining, dehydrating, and clearing which are carried out on the slide. The fixation of nuclei and cells is superior to that effected by alcohol, and there is much less shrinkage than in sections prepared by the usual methods. Inasmuch as the success of the method depends to a considerable extent upon the frozen sections being as thin as good celloidin sections, special attention is called to the points given on p. 250 in regard to the technique of frozen-section cutting. The method is as follows:

1. Fix pieces of tissue not more than 1 cm. thick in 4 per cent. aqueous solution of formaldehyde for fifteen to

twenty-four hours. Thicker masses of tissue should be soaked longer. The tissue may remain indefinitely in the formaldehyde solution. If the tissue is in small fragments or is too soft and friable to yield coherent sections, it may be imbedded in gelatin, as described on p. 290.

2. Trim the piece of tissue with a knife in such a manner that it will present a thickness to be frozen of not more than 5 mm. The other dimensions of the piece of tissue may be as large as the freezing box of the microtome will accommodate.

3. Rinse the piece of tissue for a few seconds in water, and place it on the freezing box of the microtome with a few drops of water beneath it; then freeze and cut frozen sections, giving careful attention to the directions for cutting such sections set down on p. 250. The sections should not be over 10 or 15 microns in thickness.

4. Float the sections off the knife into water, and select a good one. Spread this section smoothly on a slide. This is easily done by passing the slide into the water and under the section, while at the same time the section is manipulated by means of a small glass rod drawn out to a small probe-pointed extremity. The slide should be free from grease.

5. Drain off superfluous water.

6. Press the section upon the slide with a piece of smooth blotting-paper, exerting an even but not great pressure with the ball of the thumb. The section will adhere to the slide.[1]

7. Without allowing the section to become too dry, pour onto it immediately a small quantity of absolute alcohol sufficient to cover the section and adjacent parts of the slide. After a few seconds drain off the alcohol.

8. Flow from a bottle quickly and evenly over the section and adjacent surface of the slide a thin solution of celloidin

[1] If the tissue contains much mucoid material or if it has not been thoroughly fixed, the section may stick to the blotter and not to the slide. This difficulty will be overcome by covering the section with absolute alcohol for a few seconds just before blotting it, draining off, and then proceeding as indicated in this step.

in equal parts of absolute alcohol and ether, drain off immediately, blow the breath briskly once or twice on the surface of the section, and immerse the slide immediately in water for a few seconds. Thus a thin film of celloidin is formed, which fastens the section to the slide. The solution of celloidin should be almost watery in consistence, and so thin that it will form drops readily without stringing. If it is too thin, however, it will not hold the specimen on the slide, and if it is too thick, the layer on the slide will become white when the slide is immersed in water. The film of celloidin on the slide should be so thin as to be almost invisible.

9. Stain the section by any of the usual methods applied to sections affixed to the slide. The thin layer of celloidin offers no obstruction to the staining of the section. The staining fluid may be heated on the slide, as in the staining of tubercle bacilli with fuchsin, without loosening the section. The most satisfactory staining of these sections for general purposes is with alum hematoxylin, followed by eosin. The various methods for staining bacteria in tissues are applicable to these sections.

10. Dehydrate by flooding the section and adjacent portions of the slide with absolute alcohol. This will remove most of the celloidin. It will not, however, loosen the section unless its action is unusually prolonged.

11. Clear by flooding the preparation and adjacent parts of the slide with oil of origanum. If parts of the section refuse to clear, apply alcohol again and then more oil of origanum. When clear, drain off the oil, blot with a piece of *dry* blotting-paper, and mount in balsam. When mounted, none of the cellodin will be visible if the proper technique has been employed.

The staining fluids, alcohol, and oil of origanum are conveniently applied from drop bottles.

Alcohol.—The strength of alcohol ordinarily used in laboratories is 95–96 per cent. Absolute alcohol is much more expensive. Tissues hardened in either of these strengths shrink a great deal. The exposed surface becomes extremely hard, and the outer layers of the cells of

tissues, like a rabbit's kidney, for example, are as shrunken and flattened as though dried in the air. It is only inside of this hard casing, where the alcohol has penetrated more slowly and has been somewhat diluted by the fluid of the tissue, that the cells are better preserved. Moreover, this extreme hardening of the surface hinders the penetration of the alcohol into the deeper parts.

Tissue which is to be hardened in absolute or 95 per cent. alcohol should be cut into thin pieces, preferably not over ½ cm. thick. The volume of alcohol used for hardening should be fifteen to twenty times as great as the specimen, and should be changed after three or four hours. The tissue should be kept in the upper part of the alcohol by means of absorbent cotton, or the jar may be frequently inverted and the alcohol thus kept of even strength.

The advantages of strong alcohol, 95 per cent. and absolute, are that the tissue is more quickly fixed than with weaker strength, and that at the same time it is made quite hard—a quality more necessary formerly than now when tissues are so generally embedded. Tissues hardened in strong alcohol should later be transferred to 80 per cent. alcohol for preservation, or the staining properties will gradually become impaired.

For general purposes it will be found better to place tissues at first into 80 per cent. alcohol, which should be replaced in two to four hours by 95 per cent. alcohol. In this way less shrinkage is caused and the surface of the tissues is not made so hard.

Tissues which have been fixed in Zenker's and other fluids should, after thorough washing in running water, be placed directly in 80 per cent. alcohol for further preservation. Change the alcohol occasionally as it becomes cloudy.

Corrosive Sublimate.—Use a saturated solution (made by heat) in normal salt solution. The addition of 5 per cent. of glacial acetic acid is usually advisable. 1. Harden thin pieces of tissue (2 to 5 mm.) for six to twenty-four hours. 2. Wash in running water twenty-four hours. 3. Preserve in 80 per cent. alcohol.

Tissues hardened in corrosive stain quickly and brilliantly in nearly all staining solutions. It is the only fixative after which the Heidenhain-Biondi triple stain gives good results.

Giemsa's Corrosive Sublimate-alcohol Fixative.—

Saturated aqueous solution of corrosive sublimate,	2 parts;
Absolute alcohol,	1 part.

This fixative is absolutely necessary for tissues which are to be stained by Giemsa's method. It requires at least forty-eight hours, and is to be renewed after twenty-four hours. The tissue may remain as long as three months in the solution without disadvantage if evaporation is prevented.

Flemming's Solution.—

Osmic acid, 2 per cent. aqueous solution,	4;
Chromic acid, 1 per cent. aqueous solution,	15;
Glacial acetic acid,	1.

1. Fix in the solution one to three days. 2. Wash in running water six to twenty-four hours. 3. Alcohol, 80 per cent.

It is best to keep the osmic acid in a 2 per cent. solution and the chromic acid in a 1 per cent. solution. The mixture can then be quickly made up fresh at the time it is needed. The best stains after hardening in Flemming are Babes' safranin, aniline-gentian-violet, and carbol-fuchsin.

Pieces of tissue for hardening in Flemming's solution should not be over 2 mm. in thickness, because it has very slight penetrating properties.

Hermann's Solution.—

Osmic acid, 2 per cent. aqueous solution,	4;
Platinic chlorid, 1 per cent. aqueous solution,	15;
Glacial acetic acid,	1.

This modification of Flemming's solution is perhaps an even better fixative than the model on which it is based, but is more expensive. It should be employed in the same manner.

Pianese's Solution.—

Chlorid of platinum and sodium, 1 per cent. aqueous solution (platinic),	15 c.c.;
Chromic acid, $\frac{1}{4}$ per cent. aqueous solution,	5 "
Osmic acid, 2 per cent. aqueous solution,	5 "
Formic acid, C. P.,	1 drop.

Fix small pieces of tissue, not over 2 mm. thick, in the solution for thirty-six hours. Wash in running water for twelve hours, then 80 per cent. alcohol. Stain paraffin sections by Pianese's special methods (see p. 304).

This fixative and the special staining methods are particularly recommended for the study of karyomitosis and of the so-called cancer bodies.

Boiling.—Boiling precipitates the soluble albumin in tissues as a granular material which can be readily recognized. The method is used particularly for the demonstration of albumin in renal diseases and in edema of the lungs. By means of boiling the quickest permanent mounts of tissues can be obtained. The method is not advocated on account of the shrinkage caused by the heat, but will sometimes be found useful.

Small pieces of tissue not over 1.5 cm. in diameter should be dropped into the boiling water for one-half to two minutes; cool quickly in cold water, and make frozen sections, or put into 80 per cent. alcohol. Any stain may be used; methylene-blue will be found excellent.

Müller's Fluid.—

Bichromate of potassium,	2 to 2.5	grams;
Sulphate of sodium,	1	gram;
Water,	100 c.c.	

Harden tissues six to eight weeks. Change the fluid daily during the first week; once a week thereafter. Ordinary tissues are then washed in running water overnight before being placed in alcohol. Nervous tissue is transferred directly from the fluid to the alcohol.

This famous hardening solution seems destined before long to give way entirely to better fixatives. It hardens tissues slowly, evenly, and with little or no shrinkage, but it is a poor nuclear fixative, and does not encourage any great variety of stains. The sulphate of sodium seems to serve absolutely no function. For ordinary tissues it is being replaced by Zenker's and Orth's fluids, both of which fix very quickly, besides having all its good qualities. For nervous tissues formaldehyde followed by other solutions of the chrome salts is a great deal quicker and better.

Tellyesniczky has recently recommended the following mixture, which has met with considerable favor, and which may be regarded as an improved Müller's fluid:

Bichromate of potassium,	3 parts;
Water,	100 "
Glacial acetic acid,	5 "

Fix thin sections for one to two days; thicker sections longer. Wash out thoroughly in running water. Dehydrate in graded alcohols.

Marchi's Fluid.—

Müller's fluid,	2 parts;
Osmic acid, 1 per cent. aqueous solution,	1 part.

Place small pieces of tissue in the mixture for five to eight days, wash thoroughly in running water, and harden in alcohol. For its application to degenerated nerve-fibers see page 373.

DECALCIFICATION.

Tissues which are to be decalcified should be sawn with a fine hair-saw into thin slices, so that they will decalcify quickly. It is usually desirable to saw the tissue into pieces of proper size for imbedding in celloidin. Very dense bone ought not to be over 2 or 3 mm. thick; softer tissues do not need to be thinner than 4 to 6 mm. In cutting sections after decalcifying and imbedding it is necessary to throw away the first half-dozen sections or so, because the tissue is so lacer-

ated to a slight depth by the movement of small fragments of bone in the process of sawing as to be useless for microscopic purposes. The extent of the decalcification may be tested at any time by thrusting a needle into the tissue, but it is best to avoid such a test because, of course, it tends to produce injury to the tissue.

The following steps in the decalcification of tissues must be carefully borne in mind.

1. The tissues must first be thoroughly hardened. The three most useful reagents for this purpose are alcohol and Zenker's and Orth's fluids. After the two latter reagents the tissues must have been washed thoroughly in water and placed in alcohol for at least twenty-four hours. They will then be ready for decalcification.

2. The decalcifying fluid must be used in large amounts, and, if necessary, be frequently changed. Decalcification should never be prolonged beyond four days if possible; twenty-four to forty-eight hours are better.

3. After decalcification the tissues must be thoroughly washed in running water for twenty-four hours to get rid of every trace of the acid.

4. The tissues finally must be hardened again in alcohol.

Of the various agents used for decalcifying bone, nitric, hydrochloric, chromic, picric, trichloracetic acids, etc., the most important is nitric acid. It acts quickly, without swelling the tissues or attacking injuriously the tissue-elements, and does not interfere to any marked degree with any subsequent staining process. Red blood-corpuscles will be found uninjured in tissues hardened in Zenker's fluid even after remaining four days in 5 per cent. nitric acid. This acid is used in dilute solution alone or in combination with phloroglucin.

Directions for Using Nitric Acid.—1. Decalcify in large quantities of a 5 per cent. aqueous solution of nitric acid, changing the solution every day for one to four days. 2. Wash twenty-four hours in running water to remove every trace of acid. 3. Harden in 80 per cent., and then 95 per cent., alcohol. Imbed in celloidin. According to

Schaffer, it is best to transfer the tissue directly from the nitric acid to a 5 per cent. solution of alum for twenty-four hours before placing in running water, so as to avoid any possibility of the tissue swelling.

Phloroglucin and Nitric Acid.—Phloroglucin is not a decalcifying agent, but is added to nitric acid to protect the tissues while allowing a stronger solution of the acid to be used than would otherwise be possible. The solution is prepared by dissolving 1 gram of phloroglucin in 10 c.c. of nitric acid. Solution takes place quickly, with generation of considerable heat. The fluid is reddish brown at first, but becomes light yellow in the course of twenty-four hours. Dilute with 100 c.c. of a 10 per cent. solution of nitric acid. This gives nearly a 20 per cent. solution of nitric acid. The process of decalcification in this fluid is extremely rapid; a few hours only, as a rule, are required. It is not advisable to dilute the solution by the simple addition of water, but by the use of less acid, because the phloroglucin must be present to the amount of 1 per cent. or it will not protect the tissues so well.

The following slower-acting solution may be found useful:

Phloroglucin,	1;
Nitric acid,	5;
Alcohol,	70;
Water,	30.

A rather deep single stain with alum-hematoxylin (either aqueous solution or Delafield's) will usually be found to give the best results with tissues decalcified with nitric acid. It is very important to leave the sections after staining in a large dish of water overnight, otherwise the stain will not be so sharp and clear.

Sulphurous Acid.—A saturated solution—about 5 per cent.—is used. It works very quickly and causes little swelling. The tissues should be carefully washed out in running water as after nitric acid. The stock solution rapidly grows weak through evaporation if the bottle in which it comes is not kept tightly corked.

Trichloracetic Acid.—A 5 per cent. solution of this acid has lately been recommended for the decalcification of bone and teeth. It acts more slowly than nitric acid, and seems to possess no advantages over it. Tissues must be washed out in running water, as after nitric acid.

IMBEDDING PROCESSES.

SECTIONS of hardened tissues can be cut with a razor by hand, or with a microtome knife after fastening the specimen in the microtome clamp either directly or between pieces of amyloid liver. Fair sections of firm tissues can often be obtained in this way. Thinner sections can be got by means of the freezing microtome, but these methods are all open to the objection that unless the tissue is very cohesive, portions of it are likely to fall out of the sections.

The best results would, therefore, naturally be expected from some imbedding process, employing a substance to infiltrate the tissues thoroughly and to hold the different parts in proper relative position even in the thinnest sections.

The two substances in common use for this purpose are celloidin and paraffin. Each has its advantages and disadvantages. Neither can be employed in pathological histology to the exclusion of the other. Paraffin affords the thinner sections, but they must be small if the best results are desired, and cannot be properly handled except when fastened to the slide. Hard tissues, like muscle, and tissues of varying consistency, like skin, are cut with considerable difficulty by the paraffin method. Staining is rather simpler than after imbedding in celloidin.

On the other hand, tissues of almost any consistency or size can be cut by the celloidin method, which is also capable of furnishing very thin sections.

Both methods of imbedding should be learned and used. Celloidin sections are especially good for general work, for studying the extent and relations of pathological processes, and for much of the finer histological work. Paraffin sections are better for the finest details of processes—for special work on special tissues.

Celloidin.—Schering's celloidin is the best preparation of gun-cotton (pyroxylin) to use. It keeps well, dissolves somewhat slowly, and gives a fairly transparent embedding mass, which is firm and tough, so that very thin sections can be cut. Other forms of gun-cotton are not so reliable; they often contain impurities and do not yield so firm an embedding mass.

Embedding in Celloidin.—The process consists in soaking the tissues for twenty-four hours to a number of days in two different solutions of celloidin. The two solutions are spoken of as thin and thick celloidin. To make thick celloidin 30 grams of the dry celloidin are dissolved in 200 to 250 c.c. of a mixture of equal parts of ether and absolute alcohol. Diluted with an equal amount of the ether-and-alcohol mixture, it forms thin celloidin.

The steps of the imbedding process are as follows: Pieces of tissue which have been properly fixed and finally preserved in 80 per cent. alcohol are first to be cut up with intelligence. They should rarely be over 2 to 4 mm. thick; for most purposes 2 mm. will be found sufficient. Pieces of this thickness will furnish a hundred sections or more, will embed more quickly than larger masses, and will be more rigid when mounted on a block. They should never be broader or longer than is necessary to show the whole of the process under study. Very thin celloidin sections cannot usually be obtained with tissues over $1\frac{1}{2}$ to 2 cm. square, and smaller dimensions are preferable. Beginners usually imbed larger pieces than are necessary.

The trimmed pieces of tissue are first hardened and dehydrated for twenty-four hours in 95 per cent. alcohol, followed by twenty-four hours in absolute alcohol; then soaked in equal parts of absolute alcohol and ether for the same length of time to prepare them for the thin celloidin. In the latter they remain at least twenty-four hours, preferably for a number of days, if at all thick, for in this solution occurs most of the infiltration with celloidin. Finally, the pieces are soaked twenty-four hours or more in the thick celloidin. They are then mounted on blocks of vulcanized fiber, ex-

posed to the air for two or three minutes till the surface hardens a little, and placed in 80 per cent. alcohol for six to twenty-four hours to allow the celloidin to harden.

This process of hardening may be shortened by placing the blocks for one or two hours in chloroform and then transferring them to 80 per cent. alcohol.

Briefly summed up, the steps of embedding in celloidin are as follows:

1. 95 per cent., followed by absolute alcohol, twenty-four hours each.

2. Ether and absolute alcohol, *āā*, twenty-four hours.

3. Thin celloidin, twenty-four hours to one or more weeks.

4. Thick celloidin, twenty-four hours to one or more weeks.

5. Mount on blocks of vulcanized fiber; dry a minute or two in the air.

6. Harden celloidin in 80 per cent. alcohol, six to twenty-four hours, or in chloroform for one to two hours, followed by 80 per cent. alcohol.

The second step may be omitted if time is pressing.

Instead of mounting directly from the thick celloidin, it is often advisable to allow the celloidin to evaporate until a firm mass is obtained. This is particularly true when very loose tissues are to be imbedded.

The simplest method is to place the pieces of tissue, which have been soaking in thick celloidin, in proper position in a glass dish and pour thick celloidin over them. The dish is then covered, but not too tightly, and the ether is allowed to evaporate for one or more days until the proper consistency of celloidin is reached, so that it can be cut out in blocks enclosing the specimens. If the ether evaporates too rapidly, place a large dish or a bell-jar over the covered dish. Mount the blocks, after they have been cut out and trimmed, by dipping the bases in thick celloidin and then pressing them on to blocks of vulcanized fiber. In two or three minutes they can be placed in 80 per cent. alcohol. After the celloidin mounts have been in 80 per cent. alcohol for six to twenty-four hours, the celloidin is of the proper

consistency for cutting. It is best to take a sharp knife or an old razor and trim the top of the celloidin down to where the first good section of the specimen can be cut; this will save considerable wear on the microtome knife.

In cutting, the microtome knife should be fastened very obliquely, so that as much of the edge of the knife as possible shall be used in making each section. The surface of the knife should be kept well wet with 80 per cent. alcohol, preferably from an overhanging drop-bottle.

If the sections curl, as often happens when they are thin, they are best flattened by unrolling them on to the surface of the knife with a camel's-hair brush just before the last edge of celloidin is cut through, as this serves to keep them fixed in place during the process. This method can be used when the simple transferring of sections from alcohol to water is not sufficient to uncurl them.

Celloidin sections can be stained by nearly all methods, without the necessity of removing the celloidin. When necessary, however, the celloidin is readily removed by placing the sections from absolute alcohol in oil of cloves or in the alcohol-and-ether mixture for five or ten minutes, and then passing them back through absolute into ordinary alcohol.

To Attach Celloidin Sections to the Slide.—A celloidin section can be fairly well attached to a slide by transferring it from water to a slide freshly washed in alcohol and dried with a cloth. The section is then to be firmly blotted with filter-paper so as to apply it closely to the slide and to remove all wrinkles. It should not be allowed to dry. A section treated in this way will ordinarily stand considerable manipulation without becoming loose.

Celloidin sections can be more securely attached by transferring them from 95 per cent. alcohol to clean slides and pouring over them ether-vapor from a bottle half full of ether. With a little practice sections can be fastened in a few seconds. Follow slowly along the edge of the celloidin, and the frills in it will soften down. Then wash the specimen with 80 per cent. alcohol to harden the celloidin.

Imbedding in Paraffin.—Paraffin imbedding is particularly useful when very thin sections are desired. To obtain the best results the pieces of tissue should be small, soft, and of uniform consistence. In pathological work it is much better to cut the sections and to stain them after they are fastened to the slide than to stain in the mass beforehand, because then a variety of stains may be used. A complete or perfect series is not so important as in embryology, but with a little care can be obtained.

The first step in the preparation of hardened tissues for the paraffin bath is to cut them into small, thin, square or rectangular pieces, not over 1 cm. square, perhaps, for the best results, and not over 2 to 3 mm. thick. It should be stated, however, that with proper skill, a heavy, sharp knife, and a rigid microtome very thin paraffin sections can be obtained with tissues measuring 4×3 cm. The pieces of tissue are then thoroughly dehydrated by soaking first in 95 per cent. and then in absolute alcohol. From alcohol they are put in some substance, such as chloroform or oil of cedar, which has the property of mixing with alcohol and of dissolving paraffin. From the chloroform they are transferred to a saturated solution of paraffin in chloroform, and then passed through two separate baths of the melted paraffin to get rid of every trace of the chloroform. If oil of cedar is used, the specimens are transferred directly from it into the melted paraffin, or they may be placed first for half an hour or so in chloroform to get rid of the oil of cedar. This procedure enables one to make use, for certain dense tissues, such as the skin, of the better penetrating powers of the oil, and yet avoid carrying it into the paraffin bath.

One advantage of the chloroform method is that the duration in the hot paraffin, the objectionable feature of the paraffin method, is shortened, because the tissues are already somewhat infiltrated with paraffin. Another advantage is that the paraffin bath purifies itself, because the chloroform rapidly evaporates. When oil of cedar is used, the paraffin must be renewed frequently.

The methods of imbedding in paraffin are briefly stated as follows:

Method No. 1.

1.	95 per cent. alcohol,	6–24 hours.
2.	Absolute alcohol,	6–24 "
3.	Chloroform,	6–24 "
4.	Chloroform saturated with paraffin,	6–24 "
5.	Paraffin bath, two changes,	2– 4 "
6.	Imbed and cool quickly in cold water.	

Method No. 2.

1.	95 per cent. alcohol,	6–24 hours.
2.	Absolute alcohol,	6–24 "
3.	Oil of cedar, two changes,	6–24 "
4.	Paraffin, three changes, until no odor of oil of cedar.	2– 8 "
5.	Imbed and cool quickly in cold water.	

Method No. 3.

1.	Acetone,	½– 2 hours.
2.	Xylol or chloroform,	15–30 minutes.
3.	Paraffin,	30–90 "

This method is recommended when there is great haste. We are not sure that it does not shrink the tissue more than the other methods. The quantity of acetone used should be at least twenty-five times the volume of the tissue. With larger amounts of tissue the acetone should be changed after thirty minutes or an hour, and a longer exposure to the acetone and paraffin may be necessary.

For imbedding paraffin specimens metallic boxes can be used, or forms made round or square from strips of sheet lead or tin. Many prefer paper boxes, which can be made easily of any size desired from stiff writing-paper.

Melted paraffin is poured into the paper box to the depth of about 1 cm. The pieces of tissue are then placed in the box with that side down from which sections are preferred. When all the pieces are arranged in order with about half a centimeter or more between them, the box is placed on the surface of a large dish of cold water, on which it floats, so

that the paraffin may cool quickly without crystallizing. Sometimes it is advisable to set the paper box with the specimens in it in the paraffin oven for a short while, so as to get rid of any bubbles carried in by the specimens. After the paraffin has hardened, the paper is removed and the paraffin is divided up according to the pieces in it. One of the blocks is then fastened to the object-holder by heating the latter in a flame until it will just melt the paraffin when the block is held in proper position against it. The holder is then quickly cooled in cold water.

The upper surface of the paraffin should now be shaved down to the specimen. The four sides are to be carefully trimmed; the upper and lower surfaces should be parallel and not cut too close to the specimen, otherwise the sections will not adhere to each other; the lateral surfaces should, as a rule, be cut close to the tissue, especially if very thin sections are desired, because if a rim of paraffin is left it is likely to cause wrinkling of the sections. The holder is finally carefully adjusted in the paraffin microtome.

To get good sections which will adhere to each other and form a ribbon the temperature of the room must be regulated to suit the degree of hardness of the paraffin used. An open window will often make all the difference needed to obtain good results. The harder the paraffin the warmer the room must be. The temperature can be raised by burning a Bunsen flame near the microtome or lowered by the presence of a lump of ice. It will often be found advantageous to dip the holder and paraffin block into ice water just before cutting sections, or to rest a small bag containing cracked ice on the block and knife for a few minutes just before cutting.

The ribbons of sections as cut, usually a slideful, are laid on the surface of a large dish of warm water at about 44° C., and if necessary gently stretched so as to remove all wrinkles. Paint the surface of a slide with a thin layer of Mayer's glycerin-albumin mixture, wipe off all excess with a towel so that only a faint layer is left, dip the slide under the sections, arrange them in order, lift the slide, and drain off the water. The slide is then placed in a slanting position

until dry, when it is put in the incubator for two to twelve hours at a temperature of about 54° C. This process attaches the sections firmly to the slide.

To get rid of the paraffin in the sections they are treated with two or three changes of xylol, and then with absolute followed by 95 per cent. alcohol.

If for any reason the celloidin-and-oil-of-cloves mixture is used for attaching the sections to the slide, the paraffin is removed by means of xylol, followed by origanum or bergamot oil, and finally by 95 per cent. alcohol, because absolute alcohol will dissolve the celloidin.

Serial Sections by the Celloidin Method.—1. For Tissues in General.—With a little care perfect serial sections can be made by the following method, and each slide of sections can be stained in whatever way seems best. The specimen is imbedded, mounted on vulcanized fiber, and hardened in 80 per cent. alcohol in the usual way. In cutting moisten the microtome knife with 95 per cent. alcohol. As the sections are cut they are drawn up on the surface of the knife and arranged in regular order by means of a camel's-hair brush until a slideful is ready. They are then drawn on a clean and numbered slide held against the back of the knife. After being carefully arranged the sections are fastened to the slide by means of ether-vapor (see p. 283) poured over them from a half-full bottle. Care must be taken that every edge of the celloidin is fully softened down. The slides are then placed in a jar of 80 per cent. alcohol to be stained at leisure.

2. Another method, often convenient where the stain is of little importance, is as follows: The tissue is stained, in bulk, in alum-cochineal or some other staining fluid that will penetrate, and then imbedded in celloidin in the usual way. After being mounted on vulcanized fiber the specimen is hardened in chloroform instead of in 80 per cent. alcohol. From the chloroform the specimen is transferred to oil of thyme. After it is thoroughly penetrated by the latter it is ready to be cut. The knife is to be moistened with oil of thyme. The sections as cut are arranged on the knife, and then trans-

ferred to slides placed against the back of the knife. The slides covered with sections can be placed under a bell-jar as fast as they are ready until all are cut, because the oil of thyme evaporates slowly. Balsam and cover-slips can be added after the cutting is finished.

3. Darkschewitsch has recently proposed a comparatively simple method for preparing a series of celloidin sections. Take a glass cylinder without a neck, of about the diameter of the specimen to be cut. Prepare a series of circles of filter-paper cut of a size just to fit the bottle, number in order, and wet them with alcohol. Each section is removed from the microtome knife by pressing one of the paper circles upon it and drawing it off. The paper is then inverted so that the section is uppermost, and deposited in proper order in the bottle, where the series forms a column, each section resting upon a numbered paper. The sections can be kept indefinitely by filling the bottles with 80 per cent. alcohol. When ready to stain, the alcohol is poured off, the sections washed with water if necessary, and then the staining solution poured into the bottle. Other reagents are used in the same manner, or sections can be treated with the reagents in flat plates, as they do not readily slip off the papers.

4. Weigert's method for a series of celloidin sections was designed especially for the nervous system and is rather complicated. The process depends on transferring the sections as cut to narrow strips of tissue-paper. To do this each section as cut is arranged in proper position close to the edge of the knife. Then a strip of tissue-paper twice as wide as the section is gently placed upon it, and the sections withdrawn from the knife. The success of the process depends on having but little alcohol on the knife, otherwise the specimen will not stick. Each specimen is placed on the paper to the right of the last one. The strips of paper when full are kept moist by being placed with the specimens uppermost on a moist surface composed of a layer of blotting-paper wet with alcohol, covered with a sheet of tissue-paper, and lying in a shallow dish.

When all the sections have been cut, each strip of them is taken in turn and coated on both sides with a thin film of celloidin in the following way: A strip of sections with the specimens below is first pressed gently down upon the surface of a slide covered with a thin layer of celloidin. This fastens the sections and the paper can be removed. Then a thin coat of celloidin is poured over the sections and the slide is placed on its edge to drain. When the surface of the celloidin is dry, the strips can be marked by a fine brush dipped in methylene-blue. As soon as the slides are placed in the staining solution the celloidin peels off, taking the specimens with it. Later, the strips of specimens can be divided as desired. On account of their thickness they should be cleared, after dehydrating in 95 per cent. alcohol, in a mixture of xylol 3, carbolic-acid crystals 1.

5. F. H. Verhoeff recommends this method:

In cutting the sections, the knife is not carried entirely through the celloidin block, but an uncut edge, about 3 mm. wide, is left each time. After twenty or more sections are cut in this way the knife is carried all the way through, thus producing a little book of sections. It is probably most convenient to keep each book in a separate bottle; but no difficulty is usually experienced in determining the proper order after the sections are mounted. Another way to keep them in order is to string them on a silk thread through their uncut margins. In beginning a new book a wider margin should be left for the first one or two sections, as otherwise the sections may not adhere, or the first section may be cut at double thickness. Each book is stained in the same manner as a single section, except that it is best to use slow-acting stains, so that the staining will be uniform throughout. The individual sections are not separated until the book is in alcohol preliminary to clearing. Then each section is either torn off with forceps, or the book is taken up on cigarette paper and the uncut margin removed with scissors. Each section in order is then removed, cleared quickly in oil of origanum, and placed on a slide.

6. Suzuki recommends spreading the sections out on a slide or glass plate, blotting the celloidin at one corner of the section, and marking the number of the section on it with a certain Japanese or Chinese ink by means of a fine-pointed brush. It is said that the solid India ink freshly rubbed up with a little water is satisfactory for the purpose. The sections are placed in 80 per cent. alcohol after marking.

To obtain **serial sections by the paraffin method** it is only necessary to avoid losing any of the sections from the ribbon as ordinarily cut. Perhaps the easiest and safest way is to cut long ribbons, a yard or more in length, and to place them on sheets of paper in proper order. They can then readily be divided by means of needles into short series of any desired number of sections, and fastened to numbered slides by means of albumin fixative.

Wright's Imbedding Method for Frozen Sections.—This method has been devised for the purpose of holding together, in one sheet, frozen sections of small fragments of tissue, such as curettings from the uterus, so that they may be manipulated and mounted as one section by the method for frozen sections described on page 271. It depends on the fact that gelatin exposed to formaldehyde becomes insoluble in water. The method consists in soaking the fragments of tissue for a few minutes or longer in a warm 20 per cent. solution of gelatin in water, then placing the fragments close together in a watch-glass with enough of the melted gelatin to form a layer about 5 mm. thick, and allowing the gelatin to solidify, after which the block of gelatin inclosing the fragments is cut out of the watch-glass and placed in a 4 per cent. aqueous solution of formaldehyde for from eighteen to twenty-four hours. Sections are then cut from the mass on the freezing microtome, floated out in water, and manipulated and stained as described on page 250. The sections appear as sheets of gelatin inclosing sections of the fragments of tissue. The gelatin may be constantly kept on hand ready for use in corked test-tubes, each containing 8 or 10 c.c. To prevent the growth of molds in the gelatin, a little thymol or carbolic acid may

be added to it. Fragments of tissue already fixed in formaldehyde may also be imbedded in the gelatin as above described. The solidification of the gelatin in the watch-glass may be hastened by placing the watch-glass in ice-water. The action of the formaldehyde on the gelatin may be hastened by placing the vessel containing the solution of formaldehyde and the gelatin block in the paraffin oven at about 55° C. for some hours. If it is desired to do this, it is necessary that the gelatin block first be kept at room-temperature in the formaldehyde solution for at least an hour, in order to prevent the melting of the gelatin.

STAINING SOLUTIONS.

Hematoxylin and Hematein Stains.—The active coloring agent in most hematoxylin stains is hematein, which is gradually formed in the ordinary solutions from the hematoxylin by oxidation, a process occupying a number of days or weeks and spoken of as "ripening." The selective staining power of alum-hematoxylin solutions is due to the combination of this hematein with alumina. The resulting blue-colored solution is precipitated in the tissues (chiefly in the nuclei) by certain organic and inorganic salts there present, as, for instance, phosphates.

Mayer and Unna have shown that it is possible to oxidize and to ripen in an instant a solution of alum and hematoxylin by adding to it a little peroxid of hydrogen neutralized by a crystal of soda.

By employing hematein or its ammonium salt, instead of hematoxylin, Mayer has been able to obtain immediately ripened solutions which compare fairly favorably with old and well-known solutions prepared from hematoxylin by the slow process of ripening. They do not stain any better, however, and it is doubtful if, for the present at least, they become generally accepted.

Most solutions of alum and hematoxylin are not stable. A continuous chemical change is the formation from hematoxylin, by oxidation, of hematein, which, uniting with the

alum, gives a bluish or purplish solution. The degree of blueness depends largely on the freshness of the alum. As the solution becomes older free sulphuric acid is gradually formed from the alum, causing the solution to lose its bluish or purplish tint and to become reddish. A third chemical change is the continuous formation of a precipitate due to the further oxidation of the hematein, in consequence of which it is always necessary to filter alum-hematoxylin solutions just before they are used.

More alum than is needed to combine chemically with the hematoxylin is always added to the solution, for the reason that it acts as a differential decolorizer, limiting the stain largely to the nuclei of the cells. As alum-hematoxylin solutions become older they stain more quickly, but also more diffusely. This diffuseness of staining can be counteracted by adding enough alum-water to make the stain precise again. A good alum-hematoxylin solution ought not to stain the celloidin in which the section is imbedded. If the celloidin stains more or less deeply, it shows that the solution requires more alum.

Aqueous Alum-hematoxylin Solution.—

Hematoxylin crystals,	1;
Saturated aqueous solution of ammonia alum,	100;
Water,	300;
Thymol,	a crystal.

The hematoxylin crystals are dissolved in a little water by the aid of heat. The combined solution is exposed to the light in a bottle lightly stoppered with a plug of cotton. The solution will be ripened sufficiently for use in about ten days, after which time it should be kept in a tightly stoppered bottle. The solution is very easily prepared, gives beautiful results, and will keep at its best for two to three months. The proportions of alum and of hematoxylin are the same as in Delafield's solution. For Zenker preparations, which stain very slowly, it will be found more convenient to omit the 300 c.c. of water in the above formula.

Delafield's Hematoxylin.—

Hematoxylin crystals,	4 grams;
Alcohol, 95 per cent.,	25 c.c.;
Saturated aqueous solution of ammonia alum,	400 "

Add the hematoxylin dissolved in the alcohol to the alum solution, and expose the mixture in an unstoppered bottle to the light and air for three to four days.

Filter, and add—

Glycerin,	100 c.c.;
Alcohol, 95 per cent.,	100 "

Allow the solution to stand in the light until the color is sufficiently dark, then filter and keep in a tightly-stoppered bottle. The solution keeps well and is extremely powerful. So long as it is good the solution has a purplish tinge.

It would seem advisable, both in this solution and in Ehrlich's, to combine the alum, hematoxylin, and the water, and to ripen the solution for two or three weeks before adding the other ingredients which have a tendency to prevent oxidation. A fully ripened solution would then be obtained more quickly and surely.

Harris's Hematoxylin.—

Hematoxylin,	1 gram;
Alcohol,	10 c.c.;
(Dissolve the hematoxylin in the alcohol.)	
Alum (ammonium or potassium),	20 grams;
Distilled water,	200 c.c.

Dissolve the alum in the water by the aid of heat, and add the hematoxylin solution. Bring the mixture to a boil as rapidly as possible, and then add a half gram of mercuric oxide. The solution at once assumes a dark purple color. As soon as this occurs, remove the vessel containing the solution from the flame, and cool by plunging at once into a basin of cool water. As soon as cool, the solution is ready

for staining. This solution keeps for years in a well-stoppered bottle (Harris).

The addition of 4 per cent. of glacial acetic acid increases the precision of the nuclear staining.

This stain is especially adapted for sections fixed in Zenker's fluid.

Mayer's Hemalum.—

Hematein, or its ammonia salt,	1 gram;
90 per cent. alcohol,	50 c.c.;
Alum,	50 grams;
Water,	1000 c.c.;
Thymol,	a crystal.

Dissolve the hematein or its ammonia salt in the alcohol by the aid of heat, and add it to the alum dissolved in the water. The solution can be diluted with 20 parts of water or of weak alum solution.

Mayer's Acid Hemalum is prepared by adding 2 per cent. of glacial acetic acid to the above solution. The acid stain is more precise than the alkaline.

Mayer's Glycerin-alum-hematein Solution.—According to Mayer's latest investigations, glycerin is the only reliable preservative of hematein solutions. Unfortunately, it slows the staining power to a considerable extent and makes the stain less precise. He recommends the following solution for its keeping properties:

Hematein,	0.4 grams
(dissolve by rubbing up in a few drops of glycerin);	
Alum,	5 grams;
Glycerin,	30 c.c.;
Water,	70 "

Weigert's Alcohol Hematoxylin.—

Hematoxylin crystals,	10 grams;
Alcohol (absolute or 95 per cent.),	90 c.c.

The solution ripens in a week or two to a brown color, and keeps perfectly for a long time. It is used only in the

Weigert stain for myelin sheaths, for which purpose it is diluted at the time of using with water and combined with carbonate of lithium (see page 363).

Mallory's Phosphomolybdic Acid Hematoxylin.—

Hematoxylin crystals,	1.75 grams;
Phosphomolybdic acid crystals,	1 gram;
Water,	200 c.c.

The hematoxylin will dissolve almost immediately if powdered, or it may be dissolved in water by the aid of heat. The solution must be exposed to the light in a bottle plugged with cotton for five to six weeks before it is fully ripened. It will keep for several months, and can be used over and over. It is employed for staining the nervous system and connective tissue.

This stain was useful after fixation in Müller's fluid. It does not give very satisfactory results after formaldehyde followed by Weigert's quick mordants.

Mallory's Phosphotungstic Acid Hematoxylin.—

Hematein ammonium,	0.1 gram;
Water,	100 c.c.;
Phosphotungstic acid crystals (Merck),	2 grams.

Dissolve the hematein in a little water by the aid of heat, and add it after it is cool to the rest of the solution; no preservative is required. If the solution stains weakly at first, it may be ripened by the addition of 5 c.c. of a $\frac{1}{4}$ per cent. aqueous solution of permanganate of potassium, or it may be allowed to stand for a few weeks until it ripens spontaneously.

Hematoxylin may be used instead of hematein ammonium, but requires 10 c.c. of the permanganate solution to ripen it.

This staining solution will be found particularly useful for the demonstration of fibrin and of neuroglia, fibroglia, and myoglia fibrils. It also brings out with great sharpness and faithfulness of detail the structures in mitosis, including the spindles and centrosomes.

Carmine Stains.—The active staining principle in carmine solutions is carminic acid. In cochineal carminic acid is combined with an alkaline base. Carmine itself is a commercial compound containing carminic acid combined with aluminum and calcium. Carminic acid itself does not stain, but it forms compounds with certain metals, mainly with the aluminum contained in alum, which have selective staining properties.

All of the alkaline and acid solutions made with carmine owe their staining properties to carminic acid combined with the aluminum, and perhaps also to the calcium contained in the carmine.

Alum Carmine.—

Carmine,	2 grams;
Alum,	5 "
Water,	100 c.c.

Boil twenty minutes, adding enough water to make up for that lost by evaporation. When cool, filter and add a crystal of thymol to prevent the growth of mould.

Alum Cochineal.—

Powdered cochineal,	6 grams;
Ammonia alum,	6 "
Water,	100 c.c.

Boil for half an hour; add water to make up for that lost by evaporation. Filter and add a crystal of thymol.

Mayer's Alcoholic Carmine (Paracarmine).—

Carminic acid,	1.0 gram;
Chlorid of aluminium,	0.5 "
Chlorid of calcium,	4.0 grams;
70 per cent. alcohol,	100.0 c.c.

Dissolve cold or warm; allow to settle, then filter. After staining, wash out in 70 per cent. alcohol to which is added 2.5 per cent. glacial acetic acid if a more purely nuclear stain is desired.

Orth's Lithium Carmine.—

Carmine,	2.5 to 5 grams;
Saturated aqueous solution of carbonate of lithium,	100 c.c.;
Thymol,	a crystal.

The carmine dissolves at once in the cold solution. When used as a counter-stain for bacteria in the Gram-Weigert method this solution should be carefully filtered, because organisms occasionally grow in it and may give rise to confusion in the stained preparations.

Neutral Carmine.—Dissolve, without heating, 1 gram of best carmine in 50 c.c. of distilled water plus 5 c.c. of strong aqua ammoniæ. Expose the fluid in an open dish until it no longer smells ammoniacal (about three days); then filter and put away in a bottle for future use. The odor of the solution will soon become bad, but the staining properties will remain unaffected.

Aniline Dyes.—It is extremely important that all aniline dyes used in histological work should be obtained, with possibly a few exceptions, from Grübler, or from the firms manufacturing the dyes, either directly or from their authorized agents. In no other way is it possible to obtain with certainty the results expected. In this country Eimer & Amend, of New York City, are the chief agents for Grübler.

Aniline dyes come in the form of a powder or as crystals, and most of them keep well in that condition. Methylene-blue for one, however, seems to be an exception. After the original package has been opened for a short while the dye is said to lose in intensity of staining power. It is well to keep on hand saturated alcoholic solutions of certain of the dyes, because they keep well in that form, and are ready for use when a saturated alcoholic solution is wanted. This is particularly true of methylene-blue, fuchsin, and methyl-violet.

Aniline dyes are derived from either aniline or toluidin, or from both together. They may be regarded as salts having basic or acid properties. The basic colors stain cell-nuclei,

including bacteria, for which they show a marked affinity. The acid colors stain diffusely. The basic dyes most commonly employed in pathological histology are methylene-blue, fuchsin, methyl-violet, and safranin. Of the acid colors, eosin, picric acid, and acid fuchsin are most in use.

As a rule, every aniline dye has one or more standard solutions which are used largely to the exclusion of others, for the reason that, being required for certain purposes, they are kept in stock. As they are thus always at hand, they are used where simple solutions might be used. For instance, Löffler's methylene-blue solution is often used, because ready and convenient, when a simple aqueous solution would do as well.

In the following pages we have arranged under each dye the solutions of it most in use:

Methylene-blue.—1. Saturated solution in 95 per cent. or absolute alcohol. A stock solution to be used in making other solutions. It can be used as a stain by adding 1 part to 9 parts of water.

2. Aqueous solutions of various strengths are often used, and can be made up as needed.

3. *Löffler's Methylene-blue Solution.*—

Saturated alcoholic solution of methylene-blue,	30 c.c.;
Solution of caustic potash in water, 1 : 10,000,	100 "

This is one of the most useful of the aniline staining solutions, and will keep for a long time without losing much in staining power.

4. *Kühne's Methylene-blue Solution.*—

Saturated alcoholic solution of methylene-blue,	10;
5 per cent. carbolic-acid water,	90.

This is a stronger staining solution than Löffler's, but the resulting stain does not seem so sharp and clear.

5. *Gabbet's Methylene-blue Solution.*—

Methylene-blue,	2;
Sulphuric acid,	25;
Water,	75.

It is used as a decolorizer and contrast-stain for tubercle bacilli.

6. *Unna's Alkaline Methylene-blue Solution.*—The strongly alkaline solution of methylene-blue recommended by Unna for staining plasma-cells has been found extremely valuable as a general stain in connection with eosin, which should be used first. The solution should be diluted 1 : 10, or 1 : 5, for staining; it stains better after ripening for a week or two:

Methylene-blue,	1;
Carbonate of potassium,	1;
Water,	100.

(For method of using see page 336.)

7. *Unna's Polychrome Methylene-blue Solution.*—The polychrome methylene-blue solution, much used by Unna in various staining methods, is an old alkaline solution of methylene-blue, of which the one on page 298 is the original formula, in which, in consequence of oxidation, methyl-violet and methylene-red have formed. Months are required for the process of oxidation to take place. The ripened solution may be obtained from Grübler.

8. *Sahli's Borax Methylene-blue Solution.*—

Saturated aqueous solution of methylene-blue,	24;
5 per cent. solution of borax,	16;
Water,	40.

Mix, let stand a day, and filter.

Fuchsin.—1. Saturated alcoholic solution to be kept in stock.

2. *Ziehl-Neelson's Carbol-fuchsin.*—

Saturated alcoholic solution of fuchsin,	10 c.c.;
5 per cent. carbolic-acid water,	90 "

This solution is very powerful, stains quickly, keeps well, and can be employed for a variety of purposes.

3. *Verhoeff's Carbol-fuchsin Solution.*—

Carbolic acid, melted,	25 c.c.;
Absolute alcohol,	50 "
Fuchsin,	2 grams.

Combine the ingredients and place over night in an incubator to ensure complete solution; cool and filter. This stock solution of carbol-fuchsin, unlike the dilute aqueous solution which slowly deteriorates, is permanent and does not even require to be filtered again.

For use in staining coverslip preparations, add two drops of this stock solution to eight drops of distilled water. When larger quantities of staining solution are required, the dilution is made in the proportion of 1 c.c. of the stock solution to 6 c.c. of distilled water.

4. *Aniline-fuchsin.*—

Saturated alcoholic solution of fuchsin,	16 c.c.;
Aniline-water,	84 "

Methyl-violet.—1. Aqueous solutions of various strengths, ½ to 2 per cent., keep well and are used for staining nuclei, bacteria, and amyloid.

2. Methyl-violet can be used instead of gentian-violet in Ehrlich's solution. Weigert recommends two permanent stock solutions by means of which the aniline methyl-violet solution can be made up easily when wanted.

Solution 1.—Absolute alcohol,	33;
Aniline,	9;
Methyl-violet in excess.	

Solution 2. Saturated aqueous solution of methyl-violet.

The staining solution consists of—

Solution 1,	1;
Solution 2,	9.

This mixture will keep at the most for fourteen days.

3. For staining neuroglia-fibers Weigert employs a saturated solution made with the aid of heat in 70–80 per cent. alcohol.

Gentian-violet.—This dye is not a definite chemical substance, but a mixture of crystal-violet, methyl-violet, and dextrin. It is better to discard it entirely, and to use methyl-violet instead in the staining solutions given; they are cited here as originally given only because they are classical.

1. Saturated alcoholic solution to be kept in stock.

2. *Ehrlich's Aniline-gentian-violet.*—

Saturated alcoholic solution of gentian-violet,	16 c.c.;
Aniline-water,	84 "

During the first few hours after the solution is made considerable precipitation takes place, so that it is best not to use it for twenty-four hours. After about ten days it begins to lose its staining power. (See under methyl-violet, page 300.)

Zenker recommends a solution without alcohol: Dissolve the gentian-violet directly in the aniline-water. The color is said to be less easily removed from tissues when this solution is used.

3. *Stirling's Solution of Gentian-violet.*—

Gentian-violet,	5 grams;
Alcohol,	10 c.c.;
Aniline,	2 "
Water,	88 "

This solution is said to keep remarkably well.

4. *Carbol-gentian Violet.*—

Saturated alcoholic solution of gentian-violet,	10 c.c.;
5 per cent. carbolic-acid water,	90 "

Safranin.—Two of the many preparations by this name have been found especially useful:

1. Safranin O soluble in water.
2. Safranin soluble in alcohol.

The three following solutions of safranin can be thoroughly recommended:

1. Saturated aqueous solution of "safranin O soluble in water" (to be made with the aid of heat).

2. A mixture of equal parts of—

   A saturated aqueous solution of "safranin O soluble in water."

   A saturated alcoholic solution of "safranin soluble in alcohol."

3. *Babes' Aniline Safranin.*—

2 per cent. aniline-water,	100;
"Safranin O soluble in water,"	in excess.

Saturate the solution by heating it in a flask set in hot water to 60–80° C.; filter.

This solution is extremely powerful, stains almost instantly, and will keep about two months.

Bismarck Brown.—The most common solutions are the following:

1. A 1 per cent. aqueous solution.

2. A saturated aqueous solution made by boiling (3–4 per cent.).

3. A saturated solution in 40 per cent. alcohol (2–2½ per cent.).

Unlike other aniline colors, Bismarck brown will keep in glycerin mounts and can be fixed in nuclei by acid alcohol. The stain is not used so much as formerly, except as a contrast stain in Gram's method and for photographic purposes. Other basic stains less frequently used, and then generally in aqueous solutions, are dahlia, methyl-green, iodin-green, and thionin.

Diffuse Stains.—1. **Eosin** is sold in two forms—as "eosin soluble in water," and as "eosin soluble in alcohol." The first is to be preferred, because a greater degree of differentiation in stain can be obtained with it. Keep on hand a saturated aqueous solution, to which a crystal of thymol has been added, and dilute with water as needed. The strength of solution to be used varies somewhat with the tissue and the reagent in which it has been fixed, but generally lies between $\frac{1}{10}$ and $\frac{1}{2}$ per cent. when the eosin is used after a hematoxylin stain. These dilute solutions should contain 25 per cent. of alcohol, otherwise they will not keep well. When eosin is employed before an aniline dye, such as methylene-blue, a 5 per cent. or even a saturated solution should be taken. Solutions of eosin should always be filtered immediately before use.

2. **Picric Acid.**—Saturated alcoholic and aqueous solutions should be kept in stock, to be diluted as needed.

3. **Acid Fuchsin.**—Aqueous solutions of various strengths are used. It is advisable to keep on hand a 5 per cent.

solution and to dilute it to the strength required. A crystal of thymol should be kept in the solution because otherwise molds readily grow in it.

Altmann's Aniline Acid Fuchsin Solution.—

Acid fuchsin,	20. grams;
Aniline water,	100. c.c.

4. **Van Gieson's Picro-fuchsin Solution.**—This valuable solution was originally made by adding to a saturated aqueous solution of picric acid enough of a saturated aqueous solution of acid fuchsin to give to the fluid a deep garnet-red color, and for certain purposes, as in staining after Zenker's fluid, this strong solution is to be preferred. Freeborn has recently given more precise directions for making up the solution according to the purpose for which it is to be used.

For Connective Tissue.—(See page 338).

1 per cent. aqueous solution of acid fuchsin,	5 c.c.;
Saturated aqueous solution of picric acid,	100 "

For the Nervous System.—(See page 346).

1 per cent aqueous solution of acid fuchsin,	15 c.c.;
Saturated aqueous solution of picric acid,	50 "
Water,	50 "

Picro-nigrosin (*Martinotti*).—Dissolve picric acid and nigrosin to saturation in 70 per cent. alcohol.

Combination Stains.—**Biondi-Heidenhain Staining Solution.**—

Saturated aqueous solution of orange G,	100;
Saturated aqueous solution of acid fuchsin or rubin S,	20;
Saturated aqueous solution of methyl-green,	50.

(About 20 gm. rubin S., 8 gm. orange G., and 8 gm. methyl-green; dissolve in 100 c.c. of water.)

Make up the separate solutions and let them stand for several days with excess of coloring matter (shaking the bottles

occasionally) until they are saturated. Then mix the solutions. For staining, dilute the combined solution with water 1 : 60 to 1 : 100.

The following tests are used for finding out if the proper combination has been obtained: The addition of acetic acid should make the solution redder; a drop of the solution on filter-paper should make a blue spot with green in the center and orange at the periphery. If a red zone appears outside of the orange, then too much acid fuchsin is present.

Pianese's Staining Solutions and Staining Methods.—The following stains, devised by Pianese, are recommended by him particularly for the study of cancer, but will be found useful in many lines of histological investigation. The first two were used by him for tissues hardened in corrosive sublimate or in Zenker's fluid; the others, only after his special fixative (given on page 276). The methods are intended for paraffin sections:

I. Carmine and Picro-nigrosin.—1. Stain in neutral or lithium carmine.

2. Decolorize in acid alcohol.

3. Wash in water.

4. Absolute alcohol.

5. Aniline-gentian-violet, ten minutes.

6. Iodin solution, two to three minutes.

7. Absolute alcohol, so long as any color is discharged.

8. Saturated aqueous solution of picric acid and of nigrosin, five minutes.

9. Decolorize in a 1 per cent. alcoholic solution of oxalic acid.

10. Water, several minutes.

11. Absolute alcohol.

12. Oil of bergamot.

13. Balsam.

Nuclei, red; cell-protoplasm, light olive-green; connective tissue, dark olive-green; elastic fibers, bluish; bacteria and blastomycetes, violet.

II. **Methylene-blue and Eosin in Borax Solution.**—Keep three solutions on hand:

(*a*) Saturated solution of methylene-blue in a saturated aqueous solution of borax.

(*b*) ½ per cent. solution of "bluish eosin" in 70 per cent. alcohol.

(*c*) Saturated aqueous solution of borax.

For use mix together 2 parts of the filtered solution *a*, 1 of *b*, and 2 of *c*. The different steps of the staining process are as follows:

1. Absolute alcohol.
2. Staining solution, ten to twenty minutes.
3. Decolorize in a 1 per cent. solution of acetic acid.
4. Wash in water.
5. Absolute alcohol.
6. Xylol.
7. Xylol balsam.

Nuclei, blue; red blood-globules, cell-protoplasm, granules of eosinophiles, connective tissue, etc., rose-red.

III. *a.* **Malachite-green, Acid Fuchsin, and Nigrosin.**—

Malachite-green,	1.	gram;
Acid fuchsin,	.4	"
Nigrosin,	.1	"
Water,	50	c.c.;
Alcohol saturated with acetate of copper,	50	"

1. Absolute alcohol.
2. Stain in 20 drops of above solution diluted with 10 c.c. of distilled water for twenty-four hours.
3. Decolorize in a ½ per cent. aqueous solution of oxalic acid.
4. Wash in water.
5. Absolute alcohol.
6. Xylol balsam.

Resting nuclei, light red; protoplasm, reddish yellow. In the karyokinetic figures, nuclein green; fibrillæ of the achromatic spindle and of the mitoma, bright red; centrosome and polar bodies, red; the rest of the cell-body, a reddish-yellow color.

III. *b.* **Malachite-green, Acid Fuchsin, and Martius Yellow.**—

Malachite-green,	.5	gram;
Acid fuchsin,	.1	"
Martius yellow,	.01	"
Distilled water,	150	c.c.;
Alcohol, 96 per cent.,	50	"

1. Stain in the solution without diluting, half an hour.
2. Absolute alcohol.
3. Xylol.
4. Xylol balsam.

Nuclei of resting and dividing cells, green; cell-cytoplasm, connective tissue, etc., rose-colored; "cancer-bodies," mainly red, but in masses of them some are red and some green.

IV. **Acid Fuchsin and Picro-nigrosin.**—

Saturated alcoholic solution of acid fuchsin,	6 drops;
Martinotti's picro-nigrosin,	8 "
Distilled water,	10 c.c.

1. 70 per cent. alcohol.
2. Stain in the solution six hours.
3. Decolorize in dilute acetic acid.
4. Absolute alcohol.
5. Xylol.
6. Xylol balsam.

Resting nuclei, red; nuclein of karyokinetic figures, yellow: cell-protoplasm, dark olive-green; "cancer-bodies," mainly olive-gray, but some or portions of them may be ruby-red.

V. **Light Green (Lichtgrün) and Hematoxylin.**—

Ehrlich's acid hematoxylin,	15 c.c.
Saturated solution of Lichtgrün in 70 per cent. alcohol,	5 "
Distilled water,	15 "

1. Distilled water.
2. Stain in above mixture half an hour.
3. Wash thoroughly in several waters.
4. Alcohol.

5. Oil of Bergamot.
6. Balsam.

Nuclei, green; "cancer-bodies" take the hematoxylin stain.

VI. **Acid Fuchsin and Hematoxylin.**—

Ehrlich's acid hematoxylin,	15 c.c.
1 per cent. solution of acid fuchsin in 70 per cent. alcohol,	15 "
Distilled water,	15 "

Stain as in V.

Nuclei, red; cytoplasm, brick-red; "cancer-bodies" take the hematoxylin stain.

Orcein, a vegetable dye obtained from certain tinctorial lichens, is used mainly for staining elastic fibers. It is soluble in alcohol, and is employed either in a neutral or acid (HCl) alcoholic solution.

Iodin is the oldest of the histological stains, but is now but little used for that purpose.

The *tincture of iodin*, a saturated solution in alcohol, is used for getting rid of the precipitate of mercury formed in tissues fixed in corrosive sublimate or in Zenker's fluid.

Lugol's solution, a solution of iodin in water containing iodid of potash, is of varying strength. Iodin in this form is much used as a test for starch, amyloid, glycogen, and corpora amylacea. In Gram's stain and its modifications iodin produces some chemical change in the coloring material employed, in consequence of which, when appropriate decolorizers are used, the stain remains fast in certain structures, while from others it is easily entirely extracted.

The strength originally employed by Gram for his staining method was—

Iodin,	1 gram;
Iodid of potash,	2 grams;
Water,	300 c.c.

Weigert in his modification of this method employed a stronger solution:

Iodin,	1 gram;
Iodid of potash,	2 grams;
Water,	100 c.c.

Recently he has recommended the following strength both for fibrin and for neuroglia-fibers:

Iodid of potash,	5 grams	saturated with iodin.
Water,	100 c.c.	

The only difference in the action of the various solutions probably is that the strong solution acts practically instantaneously, while the weaker solutions require some little time.

Acid Alcohol (*Orth's Discharging Fluid*).—

Hydrochloric acid,	1 c.c.;
70 per cent. alcohol,	99 "

Aniline Water (*Aniline-oil Water*).—Shake together 5 parts of aniline with 95 parts of water, and filter the resulting milky fluid. It should come through perfectly clear.

Carbolic-acid water is made in like manner by shaking together 5 c.c. of melted carbolic-acid crystals and 95 c.c. of water. The solution should be filtered.

Methyl-violet Shellac.—

Best white shellac,	10 gm.;
Alcohol, 95 per cent.,	20 to 25 c.c.;
Methyl-violet,	0.1 gm.

This solution will be found very convenient for marking important fields in mounted sections. It may be used with the circular markers made for this purpose, but a pen is just as convenient and less liable to cause injury to the preparation by pressure. The desired field is readily outlined under the low power of the microscope by a series of dots or a continuous line. The solution after drying is insoluble in xylol or water.

Kaiser's Glycerine Jelly for Mounting Scharlach R. Stains in.—

Finest French gelatin,	40 gm.;
Water,	210 c.c.;
Glycerin,	250 c.c.;
Carbolic acid crystals,	5 gm.

Soak the gelatin in the water for two hours. Add the glycerin and the carbolic acid and warm for ten to fifteen minutes, stirring all the while until the mixture is smooth. It is advised to filter through the finest spun glass laid wet in a funnel. The solution will, however, filter through filter-paper in the course of twenty-four hours if placed in the paraffin oven (temperature of about 54° C.). Glycerin jelly is much to be preferred to glycerin because the mounts are practically permanent; the cover-slips are fixed.

Mayer's glycerin-albumin mixture for attaching paraffin sections to slides is composed of equal parts of the white of egg and of glycerin. The mixture should be thoroughly beaten and then filtered, or after standing for some time can be decanted. Add 1 per cent. of sodium salicylate to prevent decomposition. Egg-albumin is dissolved by acids and alkalies, so that when such reagents are to be used the sections are best attached to the slide by some other substance. For this purpose *Schällibaum's solution*, of celloidin 1 part in 3 or 4 parts of oil of cloves, is often useful. Cover the slide with a thin layer of the solution. Arrange the sections in order on the slide and place it in the thermostat at 54° to 60° C. for several hours, or heat for a few seconds to half a minute over the flame until the oil of cloves runs together in drops. After cooling, remove the paraffin with xylol, pass through origanum oil to 95 per cent. alcohol, and proceed as with other paraffin sections.

Clearing Reagents.—The object of clearing reagents is to render certain tissue-elements more prominent than others. This result may be brought about by dilute acetic acid (2–5 : 100), which swells up the ground substance, so that nuclei, elastic fibers, fat, myelin, and micro-organisms are more distinct, or by alkalies, which destroy the cells and ground substance and leave only elastic fibers and bacteria but little changed. This method is used almost wholly for fresh tissues.

The same result is more commonly obtained by soaking the tissues in substances which by reason of their high index of refraction render the tissues more or less transparent.

Any structure which it is desirable to study is usually previously stained and thus easily rendered prominent. This second method is most applicable to hardened tissues.

For soaking and clearing the tissues a variety of reagents of different chemical properties are used. Glycerin and acetate of potash are not so much employed as formerly, because balsam mounts are more generally preferred. Of the other reagents (ethereal oils and coal-tar products), the the choice depends mainly on two factors—the kind of stain which has been employed, and the substance in which the sections have been imbedded. Many of the clearing reagents either dissolve celloidin or will not clear it from 95 per cent. alcohol, and nearly all of them will extract aniline colors more or less rapidly.

Most of the clearing reagents can be used after hematoxylin and carmine stains. For celloidin or paraffin sections stained by either of them oleum origani cretici, oil of bergamot, or the mixture of the oils of cloves and thyme is recommended in the order given.

For aniline stains the best clearing reagent is xylol, which, however, clears directly only from absolute alcohol. It can be used, however, for celloidin or other sections dehydrated in 95 per cent. alcohol by a simple method original with Welch, and lately brought into notice by Weigert. Blot the section on the slide with smooth soft filter-paper, and then pour on a few drops of xylol; repeat the blotting, followed by xylol two or three times, and the section will be found to be perfectly clear.

Oleum Origani Cretici.—Colorless to light brown in color; clears readily from 95 per cent. alcohol without dissolving celloidin; affects aniline colors slowly. Ordinary origanum oil is impure oil of thyme, and should not be used.

Oil of Bergamot.—Light green in color; clears quickly from 95 per cent. alcohol; does not dissolve celloidin, but after repeated use of the same lot of oil it will sometimes soften it a little. Affects aniline colors slowly, with the exception of eosin, which it extracts very quickly.

Oil of Cloves.—Straw-colored; clears quickly from 95 per cent. alcohol; dissolves celloidin; extracts aniline colors, especially methylene-blue.

Oil of Thyme.—Colorless; clears readily from 95 per cent. alcohol; makes sections brittle; does not dissolve celloidin; affects aniline colors.

Oil of Lavender.—Clears celloidin sections readily from 95 per cent. alcohol.

Oil of Cedar-wood.—Pale straw-color; clears from 95 per cent. alcohol, but, unfortunately, clears celloidin sections very slowly; does not affect aniline colors.

Aniline (*Aniline Oil*).—Colorless when perfectly pure and fresh, but soon oxidizes and turns brown; does not dissolve celloidin; clears readily from 70 per cent. alcohol; will clear from water by Weigert's method; extracts aniline colors slowly.

Xylol.—Colorless; does not dissolve celloidin; does not affect aniline colors; clears directly only from absolute alcohol; but will clear even celloidin sections from 95 per cent. alcohol if they be blotted on the slide, and the xylol be then poured over them; the process of blotting followed by xylol must be repeated two or three times.

Dunham's Mixture of the Oils of Cloves and Thyme.—Excellent for sections stained in hematoxylin or carmine. Not nearly so expensive as pure origanum or bergamot oil.

Oil of cloves,	1 part;
Oil of thyme,	4 parts.

Filter if cloudy; clears celloidin sections readily from 95 per cent. alcohol without dissolving the celloidin.

Weigert's Mixture of Carbolic Acid and Xylol.—

Carbolic-acid crystals,	1 part;
Xylol,	3 parts.

Recommended for clearing thick sections of the central nervous system after carmine and hematoxylin stains only. The next mixture is more used now-a-days.

Weigert's Mixture of Aniline and Xylol.—

Aniline,	2 parts;
Xylol,	1 part.

Mounting Reagents.—The reagents most generally used for permanent mounts are Canada balsam, damar, and colophonium. Canada balsam is the most expensive, the most difficult to prepare properly (unless the very high-priced solid form is employed), and the most highly colored. Damar may be obtained practically colorless. Colophonium is the cheapest, is but slightly colored, and can be highly recommended. Canada balsam has the highest index of refraction of the three, but the difference between them is slight and of no practical importance.

Canada balsam occurs in commerce as a very thick, tenacious, pale, straw-colored fluid. It should be evaporated over a water-bath to drive off all volatile substances, which might affect aniline colors, until it becomes solid and brittle on cooling. Dissolve it then in xylol, which does not affect aniline colors, to a rather thin, syrupy consistency. Two pounds of Canada balsam will evaporate to about one pound; add xylol enough to make the mixture up to two pounds. In this condition it is often called *xylol balsam.*

Canada balsam has a high index of refraction, so that tissues mounted in it become very transparent, and only those parts are visible which are stained. Other solvents of Canada balsam, such as chloroform and benzine, may be used, but cannot be recommended for sections stained with aniline colors. For tissue stained with osmic acid, *chloroform balsam*, prepared in the same way as xylol balsam, should always be used, otherwise the osmic acid stain will fade rapidly.

Damar occurs in solid masses, of which the colorless pieces should be selected. Dissolve in xylol and then filter. If the solution is too thin, evaporate to the proper consistence. The only fault to be found with damar is that the xylol solution sometimes becomes cloudy. The reason for

this cloudiness is not apparent, but it may be removed by filtering again.

Colophonium also occurs commercially in the solid form: the lightest colored masses should be chosen. Two solutions should be prepared, one in xylol for anilin dyes and other stains, and one in chloroform for osmium preparations. For Wright's blood-stain use a solution of colophonium in oil of turpentine (of the best quality).

METALLIC STAINS OR IMPREGNATIONS.

Experimental investigation has shown that certain metals can be used for staining certain tissue-elements, either because they are directly reduced from solutions of appropriate salts or because they are taken up and retained by certain tissue-elements, which are rendered prominent when the metallic salt is reduced later. The most valuable metals for this purpose are silver, gold, and osmium.

Silver is used, generally in the form of silver nitrate, to stain of a brown or dark-brown color the cement substance between epithelial and endothelial cells and the ground substance of connective tissue. The method finds its chief use in pathology in demonstrating the endothelial covering of a doubtful surface, in outlining the endothelial cells of pathologically altered blood- and lymph-vessels, in demonstrating the treponema pallidum by Levaditi's method, and in staining the ground substance of the connective tissue of the cornea when that organ is used experimentally for the study of inflammation. In combination with certain other salts, especially bichromate of potassium, nitrate of silver is much employed in the Golgi methods to stain ganglion-cells and their processes in the central nervous system.

The difficulty of the silver method lies in the fact that the salt forms with albuminous fluids granular and thread-like coagula which can easily give rise to false pictures. For this reason the method is limited almost entirely to natural surfaces, which should be washed off with water or a 2 per cent. solution of nitrate of sodium before the silver solu-

tion is applied. It is generally advisable to use the nitrate of silver in a very dilute solution, 1 : 250 or 500. The solution is allowed to act on the surface for about a minute, and is then washed off with water. The tissue is next exposed in water to the action either of sunlight or of diffuse light. The outlines of the cells soon appear as dark lines, brown to black in color. The tissue to be stained should be kept stretched, because a precipitation of the silver occurs wherever there is a fold in the surface. Although nitrate of silver penetrates but a slight distance, it is possible to stain the outlines of the endothelial cells of the lymphatics and blood-vessels as well as the ground substance of the connective tissue—in a rabbit's diaphragm, for instance—by treating the upper or lower surface with the silver solution. The thoracic organs should be removed, and then the upper surface of the tendinous portion of the diaphragm left *in situ* is exposed to the action of the silver salt in the manner already described.

The outlines of the endothelial cells of blood-vessels are usually stained by injections of the silver salt through an artery. In the same way the limits of the epithelial cells of the alveoli of the lung can be stained by injections through a bronchus.

Although generally employed in solution, nitrate of silver is sometimes used in the solid form, and for the cornea this method is preferable. Chloroform the animal, preferably a rabbit, deeply; rub the cornea with a stick of nitrate of silver hard enough to remove the surface epithelium. Allow the salt to act about ten minutes, then kill the animal, remove the eye, cut out the cornea, wash it, and expose to diffuse daylight for half an hour. It is then placed in a mixture of glycerin and water, 30 parts to 70, very slightly acidulated with acetic acid (about $\frac{1}{10}$ per cent.) for twenty-four hours, so as slightly to swell and to soften the tissues. Sections of the cornea are best made with the freezing microtome. Incise the periphery a little at four points equally distant from each other, so that the cornea will lie flat. A direct stain with alum-hematoxylin gives by all odds

the best results. The sections may be mounted in glycerin or balsam. The latter method is perhaps the better. Dehydrate the sections in 50 per cent., then in 70 per cent., alcohol, clear in aniline oil, wash with xylol, and imbed in balsam. This method avoids the shrinkage which is caused by using strong alcohol.

Gold, in the form of the simple or double chlorid, is employed to stain the cytoplasm of cells of connective tissue, and more particularly the axis-cylinders of nerve-fibers and their terminal processes. Like nitrate of silver, it acts as a fixing and hardening reagent as well as a stain. Unfortunately, it penetrates tissues but a very slight distance, and, so far as staining is concerned, is inconstant in action. Its chief use in pathology is in connection with experimental work on the cornea and in regeneration. The conditions under which the reduction of the gold salt takes place are not exactly understood, but both penetration and reduction are aided by the action of organic acids, such as formic, citric, and tartaric acids, on the tissues both before and after the treatment with the gold salt. Of the many methods proposed, the following are recommended:

Löwit's Formic-acid Method.—1. Place very small bits of fresh tissue in a mixture of formic acid 1 part, and water 1 to 2 parts, until they become transparent (a few seconds to several minutes).

2. Transfer to chlorid of gold, 1 to 1.5 parts to 100 of water, for fifteen minutes.

3. Formic acid, 1 part to water 3 parts, for twenty-four hours.

4. Concentrated formic acid twenty-four hours. Preserve in glycerin or balsam.

All the steps except the first should be performed in the dark.

Ranvier's Formic-acid Method.—1. Boil together 8 c.c. of a 1 per cent. solution of chlorid of gold and 2 c.c. of formic acid. When the solution is cold place very small bits of tissue in it for one hour, in the dark.

2. Wash quickly in water.

3. Expose to diffuse light in a mixture of formic acid 10 c.c. and water 40 c.c. Reduction takes place slowly (twenty-four to forty-eight hours).

4. Harden in 70 per cent., then 90 per cent., alcohol in the dark.

Osmic Acid (perosmic acid, osmium tetroxid) is used as a fixing reagent and for staining fat and myelin, by which it is reduced. As osmic acid is quickly reduced by organic substances, care must be taken in making up the solution. Remove the label from the sealed tube in which the acid comes, and place the tube, after cracking off one end, in a glass-stoppered bottle containing enough water to make a 2 per cent. solution. If desired, the tube can be broken after it is in the bottle by violent shaking. It should be borne in mind that osmic acid is very-irritating to the bronchial mucous membrane.

In a 1 or 2 per cent. solution osmic acid is used to stain fat in teased preparations or frozen sections of fresh tissues. In Marchi's method it is used to stain fat in tissues which have been hardened for some time in Müller's fluid. As a fixing reagent it is usually combined with other reagents, as in Flemming's solution, both for its property as a fixative and for the purpose of staining any fat present.

Preparations stained in osmic acid may be kept indefinitely in alcohol. When sections are mounted they should be cleared in chloroform, and preserved in chloroform balsam prepared in the manner described elsewhere. Xylol and other clearing reagents cause the stain to fade.

STAINING METHODS.

The purpose of staining is to render prominent the different tissue-elements, so that they may be readily recognized and studied. The constant tendency now-a-days is toward selective or differential staining methods, by which but one tissue-element will be colored to the exclusion of all others,

or at least of any element that might be confused with it morphologically. These selective stains, which really are micro-chemical color reactions, enable us to differentiate from each other with ease and accuracy cellular and intercellular elements, or pathological products which otherwise look alike.

The list given on page 318 does not pretend to be either complete or perfect in arrangement, but will give some idea of the various elements which we wish to stain. Those for which we now possess more or less perfect differential stains are printed in *italics*.

The simplest selective stain is, of course, that for nuclei, and it can be obtained with a great variety of staining reagents. The most difficult element to stain differentially, although it can be done under certain conditions with a fair amount of success, is probably the axis-cylinder and its terminal processes.

Tissue-elements and pathological products differ from each other, not only in form and consistency, but also in chemical properties. While perfect preservation of form is sufficient to distinguish certain cells or elements from each other—as, for instance, polymorphonuclear leucocytes from lymphoid cells—differentiation based on micro-chemical tests is always to be preferred when possible. A few of the tests employed are colorless, like the precipitation of mucin by acetic acid. Certain tests, like the methylene-blue or gold stain for axis-cylinders, can be applied to fresh tissues only.

Others, like the various amyloid reactions, can be obtained with fresh or hardened tissues. Most of the micro-chemical reactions, however, can be employed only with tissues which have been properly preserved. It is exceedingly important, therefore, that a tissue-element be so fixed and hardened that its peculiar chemical properties be preserved intact, otherwise a differential stain for it is impossible. Each tissue-element is a law unto itself. For example, the peculiar chemical properties of red blood-corpuscles depend on the presence in them of hemoglobin. As a differential stain of the red blood-corpuscles depends on fixing this substance in them, it

- Cell.
  - Nucleus.
    - *Nucleolus.*
    - *Resting nucleus.*
    - *Linin.*
    - Bacteria.
      - 1. Do not stain by Gram.
      - 2. *Stain by Gram.*
      - 3. *Stain by tubercle-bacillus method.*
    - *Nucleus of ameba coli.*
  - Cytoplasm.
    - Centrosome and polar bodies.
    - Granules.
      - *Mast cell.*
      - *Plasma-cell of Unna.*
      - Leucocytes.
        - *Five kinds of granules* described by Ehrlich.
      - *Nissl's granules in ganglion-cells.*
    - *Dendritic processes of ganglion-cells.*
    - *Axis-cylinder and terminal processes.*
    - *Contractile elements of striated muscle-cell.*
    - *Myoglia fibrils.*
    - *Red blood-corpuscles.*
    - *Cilia of bacteria.*
  - Cuticle.
    - *Certain dots or lines in ependymal cells.*
    - *So-called cilia in certain renal cells.*
    - *Bile-capillaries.*
- Intercellular substances.
  - *Cement substance of epithelial and endothelial cells.*
  - *Ground substance of connective tissue.*
  - *Connective-tissue fibrils and reticulum.*
  - *Fibroglia fibrils.*
  - Mucous connective tissue ; *mucin.*
  - *Elastic fibers.*
  - Intercellular substances of cartilage.
  - Ground substance of bone.
  - *Myelin.*
  - *Neuroglia-fibrils.*
  - *Clubs of actinomycetes.*
  - *Capsules of bacteria.*
- Pathological products.
  - Hyaline substances.
    - *Fibrin.*
    - *Mucin.*
    - *Amyloid.*
    - *Glycogen.*
    - Hyaline.
    - Colloid.
    - *Keratohyalin.*
    - *Eleadin.*
  - *Fat.*
  - *Hemosiderin.*
  - Hematoidin.
  - *Hemoglobin.*

is necessary to find out the chemical properties of hemoglobin, such as the fact that it is soluble in water or dilute alcohol, but not in salt solution, and that it is fixed in the red blood-corpuscles by heat, absolute alcohol and ether equal parts, corrosive sublimate, formaldehyde, bichromate of potassium, etc.

While differential stains depend in part on the chemical properties of the tissue-elements, they also depend to a certain extent on the chemical properties of the staining reagents and the decolorizers used.

Some of the tissue-elements can be stained differentially in a number of ways, sometimes after one fixing agent, sometimes after another. The simplest differential stains are those where certain tissue-elements stain directly in a given solution after they have been properly fixed. Good examples are—Ehrlich's triple stain for certain cytoplasmic granules in leucocytes, and the direct stain for elastic fibers with an acid alcoholic solution of orcein.

Other differential stains depend on the property of certain elements to hold colors they have once taken up when treated with decolorizers. The best example of this is the tubercle bacillus, which holds certain stains through various acids, or aniline hydrochlorate, followed by alcohol, and, if necessary, by a contrast-stain.

Still another varied group of elements (certain bacteria, fibrin, neuroglia-fibers, etc.) depends for a differential stain in part on changes produced in gentian- or methyl-violet by iodin, in part on the decolorizer employed for extracting the coloring reagent.

Although the steps of the various staining methods differ considerably, they may be roughly arranged in the following order:

1. Staining.
2. Differentiating.
3. Decolorizing.
4. Dehydrating.
5. Clearing.
6. Mounting.

Very often two or more of the steps are combined in one, as when aniline oil is used for decolorizing, dehydrating, and clearing sections stained for certain bacteria. Sometimes the staining process occupies more than one step, as in Weigert's myelin-sheath stain. In alum-hematoxylin the differentiating reagent, the excess of alum, is combined with the stain; in Gram's method the differentiating reagent, iodin, forms a step by itself.

NUCLEAR STAINS.

For general histological work no stain is so useful or can be so highly recommended as the eosin-methylene-blue stain after fixation in Zenker's fluid. It brings out nuclei and nuclear figures with great sharpness, while at the same time it stains the cytoplasm of certain cells so that they are easily distinguished from other cells. Next in point of general usefulness is phosphotungstic acid hematoxylin, owing to the sharpness with which it stains nuclei and centrosomes, and especially nuclear figures, including the spindle. At the same time it demonstrates certain fibrils which other methods fail to show.

For class-room work alum hematoxylin, with eosin as a contrast stain, holds its own as the best general stain for celloidin sections after a variety of fixatives, but especially after Zenker fixation.

Of the carmine stains, lithium carmine, followed by picric acid, will be found the most brilliant, generally useful, and permanent, but is useless after Zenker fixation.

Safranin gives, perhaps, the most permanent stain of any of the basic aniline dyes, and confines itself very sharply to the nuclei. It is much used after certain fixing reagents, such as Flemming's and Hermann's solutions. The Heidenhain-Biondi triple stain is useful after fixation in corrosive sublimate, but cannot be employed with celloidin sections, so that its field is limited. The other aniline dyes are used on occasion or for some definite purpose, but not so generally as those mentioned above.

Alum-hematoxylin Stains.—Most alum-hematoxylin solutions will over-stain if the sections are left too long in them. The proper time required depends on the fixing reagent used and on the degree of ripeness of the staining solution. It is therefore advisable to wash a section in water occasionally and decide from the color it has acquired if it be sufficiently stained, or to mount it in water on a slide and examine with the low power of the microscope.

The best results are obtained with alum-hematoxylin solutions by staining sections just deeply enough, washing them thoroughly in several changes of water, and leaving them in a large dish of water over night. This thorough washing is done to rid the tissues of every trace of alum or of acid, so that the color will become a clear blue and will keep indefinitely.

Many microscopists prefer to stain deeply and diffusely in an old, quickly-staining alum-hematoxylin solution, and then to employ a decolorizer. The agents most used for the purpose are alum (1 per cent. aqueous solution for one to two hours), hydrochloric acid ($\frac{1}{10}$ to $\frac{1}{2}$ per cent. aqueous solution, or even the ordinary acid alcohol), and acetic acid (1 to 3 per cent. solution) for a few seconds only. After being sufficiently decolorized the sections must be thoroughly washed in water, preferably for a number of hours, otherwise the stain will fade. The objection to this method is that a pure nuclear stain only is obtained, because the acid removes the color completely from all the rest of the tissue. Under certain circumstances, as when hematoxylin is used as a contrast-stain to fuchsin in staining for tubercle bacilli, such a sharp limitation to the nuclei is desirable.

Alum-hematoxylin stains well and quickly tissues hardened in alcohol, in corrosive sublimate, and in picric acid. It stains much more slowly tissues hardened in solutions containing chrome salts, such as Zenker's and Müller's fluids. The results obtained after celloidin imbedding are always better than after paraffin imbedding.

For counter-staining eosin will usually be found to give the most beautiful contrast, although picric acid, Van Gieson's mixture, and neutral carmine are often of service.

A good alum-hematoxylin solution should have a bluish or purplish color, and should stain celloidin very faintly or not at all.

Aqueous Alum-hematoxylin; Delafield's Hematoxylin; Harris's Alum Hematoxylin (see page 293).

1. Stain in one of the above solutions two, five, or thirty minutes, or sometimes even longer. Sections of Zenker fixed tissue usually require at least one hour.

2. Wash in several changes of water, and then leave sections, if possible, for several hours or over night in a large dish of water; or better still, wash in running tap water for ten to thirty minutes.

3. Contrast-stain, usually an aqueous solution of eosin, $\frac{1}{10}$ to $\frac{1}{2}$ per cent., for one to five minutes.

4. Alcohol, 95 per cent., two or three changes to dehydrate and to remove excess of contrast-stain.

5. Clear in oleum origani cretici or in Dunham's oils-of-cloves-and-thyme mixture.

6. Xylol balsam.

The more customary method of using Delafield's alum-hematoxylin solution is to filter a few drops of it into a dish of water and to stain sections for a long time, even over night, with the very dilute solution thus obtained. It is sometimes advisable to use the aqueous solution in the same way.

Mayer's Hemalum (see page 294).—1. Stain three to five minutes or longer.

2. Wash out in 1 per cent. alum solution until the stain is precise.

3. Wash thoroughly in several changes of water.

4. Alcohol, 95 per cent.

5. Oleum origani cretici.

6. Xylol balsam.

The staining is rather diffuse, so that it has to be washed out to some extent with alum-water. Mayer's acid hemalum is more precise, and usually does not need to be decolorized, so that the second step can be omitted.

Hemalum is used for staining tissues in bulk. Twenty-four hours are required for large pieces.

Heidenhain's Hematoxylin Stain.—1. Stain twenty-four to forty-eight hours in a simple $\frac{1}{2}$ per cent. aqueous solution of hematoxylin dissolved by the aid of heat.

2. Transfer the sections directly to a $\frac{1}{3}$ per cent. aqueous solution of simple chromate of potassium for twenty-four to forty-eight hours, changing the solution frequently until no more color is given off by the sections.

3. Wash thoroughly in water.

4. Alcohol.

5. Oil.

6. Xylol balsam.

Weigert's Iron Hematoxylin.—

Prepare two solutions:

A.	Hematoxylin,	1 gram
	Alcohol, 96 per cent.,	100 c.c.
B.	Liquor ferri sesquichlorati,	4 c.c.
	Water,	95 c.c.
	Hydrochloric acid,	1 c.c.

For use mix equal parts of *A* and *B*. The mixture is deep black and is best prepared fresh each time, although it will keep and can be used for several days.

1. Stain sections for several minutes or longer.

2. Wash in water.

3. If a counterstain is wanted, place sections for a few seconds in the following solution:

Picric acid, saturated aqueous solution,	100 c.c.
Acid fuchsin, 1 per cent. aqueous solution,	10 c.c.

4. Wash in water, alcohol, carboxylol, or other clearing reagent, balsam.

Heidenhain's Iron Hematoxylin.—This staining method is particularly useful for the demonstration of the centrosome, but also stains nuclei and a variety of other structures, according to the degree of differentiation.

1. Fix in corrosive sublimate, Zenker's fluid, or alcohol.

2. Stain very thin paraffin sections (not over 5 to 6 μ thick) in a 2.5 per cent. solution of the violet iron alum (sulphate of iron and ammonium) for three to twelve hours. The sections should be placed vertical in the solution, so that no precipitate may fall on them.

3. Wash off quickly in water.

4. Stain in a 0.5 per cent. ripened alcoholic solution of hematoxylin for twelve to thirty-six hours.

5. Wash off in water.

6. Differentiate in the iron-alum solution, controlling the results under the microscope. The section should be washed off before each examination in a large dish of tap water, which immediately stops the decolorization.

7. Wash in running water for a quarter of an hour.

8. Alcohol, xylol, xylol balsam.

A counterstain with Bordeaux R. before, or with rubin S. after, the iron stain is sometimes useful.

Mallory's Chlorid of Iron Hematoxylin.[1]—The results which can be obtained by this method are equally quick and satisfactory after all of the usual fixing reagents except, perhaps, formaldehyde.

Celloidin or paraffin can be employed for embedding.

1. Stain sections on the slide for three to five minutes in a 10 per cent. aqueous solution of ferric chlorid.

2. Drain and blot the sections; then pour over them a few drops of a freshly prepared 1 per cent. aqueous solution of hematoxylin. If all of the hematoxylin is precipitated by the excess of ferric chlorid, pour off the solution and add a fresh supply. In three to five minutes the sections will be colored a dark bluish-black.

3. Wash in water.

4. Decolorize and differentiate in a ¼ per cent. aqueous solution of ferric chlorid. The sections should be kept constantly moving in the solution. The differentiation will be complete in a few seconds to one or more minutes.

5. Wash in water.

[1] Mallory: *The Journal of Experimental Medicine*, 1900, v., 18.

6. Dehydrate in alcohol.
7. Clear in oleum origani cretici.
8. Xylol balsam.

In the above directions definite strengths have been assigned to the solutions, but they may vary greatly without affecting the result. The important point is to get the sections stained deeply, and then to decolorize slowly. The differentiation can be stopped at any moment by transferring the sections to water. Sometimes it is advisable to examine the sections under the microscope to see if enough color has been extracted.

The strength of the hematoxylin solution is unimportant; it is simply necessary to have enough hematoxylin to combine with all of the iron in and on the section. The simplest way is to dissolve by the aid of heat a pinch of the crystals in a few cubic centimeters of water. A little experience will determine about how much is needed. If a solution of hematoxylin more than one or two days old is used, the color obtained is grayish-blue, and not so bright.

This method gives a sharp, permanent, dark-blue stain to nuclei; it also stains fibrin of a grayish to dark-blue color; if the decolorization is not carried too far, the contractile elements of striated muscle are brought out very sharply. In Zenker preparations the red blood-corpuscles appear of a greenish-gray color. Connective tissue is tinted a pale yellow. The nucleus of the amœba coli stains sharply by this method.

Carmine Stains.—The ordinary carmine solutions give good nuclear stains, but of the finer details in a specimen they bring out much less than a direct alum-hematoxylin stain. They are much less used now than formerly, except as contrast-stains to bacteria and to fibrin in the methods of Gram and Weigert, for which purpose lithium carmine will usually give the best results.

Alum Carmine; Alum Cochineal (see page 296).—1. Water.

2. Stain in either of the above solutions for five to twenty minutes.

3. Wash thoroughly in water.

4. Alcohol, 95 per cent.
5. Oleum origani cretici.
6. Canada balsam.

Over-staining does not occur. The solutions cannot be recommended for tissues which stain with difficulty. When used for staining in bulk, twenty-four to forty-eight hours are required.

Lithium Carmine (see page 297).—1. Water.

2. Stain two to five minutes.

3. Transfer directly to acid alcohol, one or more changes for several minutes or more, until the sections are well differentiated.

4. Wash in water.
5. Alcohol, 95 per cent.
6. Oleum origani cretici.
7. Canada balsam.

This method gives an intense and permanent bright-red nuclear stain. Over-staining is impossible. A trace of picric acid added to the alcohol used for dehydration affords a beautiful contrast-stain.

Aniline Dyes as Nuclear Stains.—Any of the basic aniline dyes may be used as nuclear stains after the following general method:

1. Stain paraffin sections in a strong solution of the dye preferred in water or in dilute alcohol for five to thirty minutes.
2. Wash in water.
3. Dehydrate in absolute alcohol.
4. Clear in xylol.
5. Xylol balsam.

With celloidin sections use 95 per cent. alcohol, blot with filter paper, and clear in xylol.

As a matter of fact, however, certain dyes and certain solutions are generally used in preference to the others. Most of the colors are more or less affected by all clearing reagents except xylol. With paraffin sections and those from which the celloidin has been removed it is very easy to dehydrate in absolute alcohol and to clear in

xylol. With celloidin sections, however, this is impossible, because the absolute alcohol will dissolve out the celloidin, and this is usually not desirable. For celloidin sections, therefore, blot with filter paper, and then pour on xylol; repeat the blotting, followed by xylol, two or three times until the specimen is perfectly clear. Mount in xylol balsam.

In washing out the excess of color it is sometimes found advantageous to acidulate very slightly either the water or the first alcohol with acetic or hydrochloric acid. This process, if not carried too far, tends to make the nuclear stain sharper.

Safranin is one of the very best nuclear-staining aniline dyes. Tissues may be hardened in alcohol, corrosive sublimate, Flemming's, Hermann's, or Zenker's fluids. Any one of the solutions of safranin given on page 301 may be used.

1. Stain paraffin sections two to five minutes to twenty-four hours according to the staining solution and fixing reagent used.

2. Wash in water.

3. Absolute alcohol, several changes, until the section appears properly differentiated.

4. Xylol.

5. Xylol balsam.

For celloidin sections dehydrate in 95 per cent. alcohol, and clear by the xylol blotting-paper method. To render the stain more precise, a few drops of acid alcohol are sometimes added to the first alcohol.

The Eosin and Methylene-blue Stain.—This stain, used on paraffin sections of tissues fixed in Zenker's fluid, can be recommended as the very best general stain yet devised. It is a sharp nuclear stain, and, at the same time, brings out with a great deal of differentiation all the various other structures in the different tissues. It has been in constant use for many years as the routine stain for all tissues in the pathological laboratories of the Harvard Medical School and Boston City Hospital.

Fix in Zenker's fluid.

1. Stain paraffin sections in a 5 per cent. aqueous solution of eosin for twenty minutes or longer. Sometimes it is advisable to get a deeper eosin stain by placing the sections in the paraffin oven for fifteen to twenty minutes.

2. Wash in water to get rid of excess of eosin.

3. Stain in Unna's alkaline methylene-blue solution (see page 299), diluted 1–4 or 5 with water, for ten to fifteen minutes.

4. Wash in water.

5. Differentiate and dehydrate in a dish of 95 per cent. alcohol, keeping the section in constant motion, so that the decolorization shall be uniform. Control the result under the microscope. When the pink color has returned to the section and the nuclei are still a deep blue, finish the dehydration quickly with absolute alcohol.

6. Xylol.

7. Xylol balsam.

For celloidin sections use 95 per cent. alcohol, blot, and pour on xylol; repeat the last two steps until the specimen is clear.

It is important to get a deep stain with eosin, because the methylene-blue washes it out to a considerable extent. The eosin must be used first, because methylene-blue is readily soluble in an aqueous solution of eosin, and therefore is quickly extracted if the eosin is used after it, while on the other hand eosin is very slightly soluble in an aqueous solution of methylene-blue which is precipitated by any excess of eosin.

The success of this staining method has been found by Wolbach to depend on the presence of colophonium in the alcohol used for differentiation. This is present in alcohol obtained from the barrel, but not in alcohol preserved in glass. It must, therefore, be added. This is most easily done by keeping on hand a 10 per cent. solution of colophonium in absolute alcohol, and adding a few drops of it to the alcohol in which the sections are differentiated. Wolbach

has also shown that sections fixed in formaldehyde may be stained by this method, provided the amount of colophonium in the alcohol be increased to from 3 to 10 per cent.

Diffuse or contrast-stains are useful to make prominent certain of the tissue-elements left uncolored by the nuclear stain. A greater richness of detail is obtained with diffuse stains if, after rather deep staining, the sections be washed out for some time in alcohol, because certain structures possess a greater affinity than others for certain diffuse stains, and by holding them are brought out sharply.

Of the diffuse stains, eosin, picric acid, and acid-fuchsin in Van Gieson's mixture are the ones most frequently employed.

Eosin is most frequently used as a contrast to alum-hematoxylin and methylene-blue stains, but is often serviceable with alum-cochineal, methyl-violet, etc. It brings out particularly well red blood-corpuscles and smooth and striated muscle-fibers. The strength of the solutions used after hematoxylin varies from $\frac{1}{10}$ to $\frac{1}{2}$ per cent., according to the tissue and the fixative used. Zenker's preparations stain intensely in eosin, so that for them a very dilute solution is advisable. When desired as a contrast-stain to basic aniline dyes, eosin should be used first in a 5 per cent. solution, because otherwise it is likely to be washed out by the nuclear stain.

Picric acid is used for contrast with the carmine stains, more rarely with alum-hematoxylin. Striated muscle-fibers and cornified epithelium are rendered especially prominent by it. To stain with picric acid it is only necessary to add a few drops of a saturated aqueous solution to a dish of water, or of a saturated alcoholic solution to a little alcohol, and allow sections to remain in the solution for a few seconds.

Van Gieson's stain (see p. 303), a mixture of picric acid and acid fuchsin, is excellent as a contrast-stain to alum-hematoxylin, especially when it is desirable to render prominent connective-tissue fibrillæ or certain pathological products. The nuclear stain with alum-hematoxylin must be

rather deep, because the picric acid to some extent extracts or overpowers it.

1. Stain deeply in alum-hematoxylin.
2. Wash in water.
3. Stain in Van Gieson's solution three to five minutes.
4. Wash in water and dehydrate directly in
5. Alcohol, 95 per cent.
6. Oleum origani cretici.
7. Xylol balsam.

Neutral Carmine (see page 297).—Neutral carmine is a diffuse stain, and is employed more especially for the central nervous system and for bone.

Filter one or two drops of the solution into 20 c.c. of distilled water, and leave the sections in the dilute solution over night. It is advisable to place a piece of filter-paper on the bottom of the dish for the sections to rest on, otherwise they may be stained on the upper side only. In double stains with hematoxylin and carmine the sections should be stained first in the hematoxylin and then thoroughly washed in water for six to twelve hours before they are stained in the carmine. After the carmine they are again to be thoroughly washed in water.

Combination Stains.—Biondi-Heidenhain Stain (see p. 303).—Tissues must be hardened in corrosive sublimate.

1. Stain paraffin sections six to twenty-four hours with the dilute solution.
2. Wash out a little in 90 per cent. alcohol.
3. Dehydrate in absolute alcohol.
4. Xylol.
5. Xylol balsam.

It is important to place the sections directly from the staining fluid into the alcohol, because water washes out the methyl-green almost instantly.

Staining in Mass.—The staining of tissues in mass is a procedure much less employed in pathological than in normal histology, but still occasionally useful. For pathological tissues a variety of stains is generally necessary. It is

therefore much better to make a series after one of the methods described, and then to stain the sections in whatever way seems best.

For staining in bulk, only a limited number of solutions are available—either those, like alum-carmine and alum-cochineal, which do not stain beyond a certain point, or those, like lithium and borax-carmine and Heidenhain's hematoxylin, which may be decolorized so as to leave only the nuclei stained, The process of staining differs from that for sections only in the length of time required for each step. Tissues ½ cm. thick will need from one to two days in the staining solution.

MITOSIS.

For the study of karyomitosis it is important that the tissue be perfectly fresh—that is, just removed from a living animal or from one just dead—and that it be fixed in a suitable reagent as quickly as possible. The best results cannot be obtained with tissues put into a hardening fluid over half an hour after removal from a living animal. On the other hand, mitotic figures can be demonstrated in tissues which have been dead for some time (twenty-four hours or more) before being put into a fixing reagent, but the details of the figures are not so perfect as those in perfectly fresh tissues, and the figures are not so numerous, because some of them have completed their changes and can no longer be recognized. It is therefore evident that mitosis can be studied much better in tissues from the lower animals, or in tissues removed by operation from the human body, than in the organs and tissues removed at post-mortem examinations.

The choice of fixing reagents for the study of mitotic figures is important. The figures can often be demonstrated after hardening in alcohol or even in Müller's fluid, but for their careful study quicker and more perfect fixing reagents must be used. Nearly all of the reagents employed penetrate slowly, so that it is absolutely necessary for the best

results that the tissue to be hardened be cut into very thin slices, rarely over 4 mm. in thickness and preferably not over 2 mm. The amount of fixing reagent used should always be at least ten to fifteen times as great as the volume of the tissue, and should be changed if it becomes cloudy.

The most important fixing reagents are—

1. Flemming's solution.
2. Hermann's solution.
3. Pianese's solution.
4. Zenker's fluid.
5. Corrosive sublimate.
6. Orth's fluid.

The first three solutions penetrate with much difficulty, so that tissues placed in them should be especially thin. The most generally useful stain for mitosis is probably safranin. The time of staining varies with the solution used. Babes' is the quickest. The mitotic figures should be stained deeply: then, when treated with alcohol slightly acidulated with hydrochloric acid, they will retain the color, while the resting nuclei will yield up most of theirs and become very pale or even colorless. In consequence of this intense stain mitotic figures can then be very readily found.

Fixation in Zenker's fluid and staining in phosphotungstic-acid hematoxylin can be highly recommended. Centrosomes and spindles are brought out with great distinctness.

Directions for Staining Karyomitotic Figures with Safranin.—1. Stain paraffin sections five minutes to twenty-four hours, according to solution used.

2. Wash in water.

3. Wash in 95 per cent. alcohol to which are added a few drops of acid alcohol.

4. Wash in pure 95 per cent. alcohol, followed by absolute alcohol.

5. Xylol.

6. Xylol balsam.

For celloidin sections dehydrate in 95 per cent. alcohol, blot, and pour on xylol; repeat the last two steps until the

specimen is clear. Safranin can be used after any of the above fixing reagents.

Other useful stains are carbol-fuchsin and aniline-methyl-violet, used in the same way as the safranin. The Gram-Weigert method gives good results after Flemming's solution.

After fixing in corrosive sublimate mitotic figures can be demonstrated by the Biondi-Heidenhain solution, which stains resting nuclei blue-violet and mitotic figures green. After Pianese's solution his special staining mixtures should be used (see page 304). His methods are said to give beautiful results.

SPECIAL STAINS FOR CERTAIN TISSUE-ELEMENTS OTHER THAN NUCLEI.

MITOCHONDRIA.

MITOCHONDRIA[1] is a term applied to certain threadlike granules, which occur in the cytoplasm of many kinds of cells and which have received various names. They may be round or oval, rod-shaped, or in the form of filaments. They disappear quickly after death and are destroyed by acetic acid and most fixing solutions. Special fixatives and staining methods are required for their demonstration. The best method is that devised by Bensley.

A. Acetic-osmic bichromate fixation.

1. Fix very thin sections of perfectly fresh tissue (1 mm. thick) for twenty-four hours in the following solution:

Osmic acid, 2 per cent. solution,	2 c.c.;
Potassium bichromate, 2.5 per cent. solution,	8 "
Glacial acetic acid,	1 drop.

2. Wash in distilled water for one hour.

3. Dehydrate in graded alcohols, 50, 70, 95, and 100 per cent., twenty-four hours each.

[1] Cowdry, E. V., "The Relation of Mitochondria and Other Cytoplasmic Constituents in Spinal Ganglion Cells of the Pigeon," *Internat. Monatsschrift f. Anat. u. Phys.*, 1–32, xxix., 1912.

4. Imbed in paraffin.

5. Cut sections not over 4 microns thick and attach them to the slide by the albumin-water method.

B. Staining.

1. Pass sections through toluol or xylol and graded alcohols to water.

2. One per cent. solution of permanganate of potassium for thirty to sixty seconds.

3. Five per cent. solution of oxalic acid for thirty to sixty seconds.

4. Wash thoroughly in water.

5. Stain for six minutes at 60° C. in Altmann's aniline acid fuchsin solution.

Aniline water,	100 c.c.;
Acid fuchsin,	20 grams.

6. Rinse in distilled water.

7. Differentiate by dipping for an instant in a 1 per cent. aqueous solution of methyl green.

8. Drain and dehydrate directly and quickly in absolute alcohol.

9. Clear in toluol or xylol and mount in balsam. The mitochondria are stained intensely red.

MAST-CELLS.

Mast-cells are found in the tissues under a variety of conditions, both normal and pathological. They are often numerous in chronic inflammatory processes and occasionally occur abundantly in leiomyomata. Their cytoplasmic granules stain intensely, like bacteria with the basic aniline dyes, especially after fixation in alcohol or formaldehyde. In tissue preserved in Zenker's fluid they usually do not stand out prominently. In eosin-methylene-blue preparations they appear like poorly stained eosinophiles. Several methods of staining the granules are given. With Unna's stains for plasma-cells a differential color-stain is obtained for the granules of the mast-cells.

Ehrlich's Method.—*A. General Stain.*—Harden in alcohol.

1. Stain with a saturated aqueous solution of dahlia.
2. Wash out with acidified water.
3. Dehydrate in 95 per cent. alcohol, absolute alcohol, xylol, xylol balsam.

B. Specific Stain.—Only the cytoplasmic granules are stained. Harden in alcohol.

1. Stain twelve hours in—

Absolute alcohol,	50 c.c.
Water,	100 "
Glacial acetic acid,	12.5 "
Dahlia,	q.s., so that the

solution is almost saturated.

2. Wash out in 95 per cent. alcohol, absolute alcohol, xylol, xylol balsam.

C. Ehrlich-Westphal Method.—Nuclei red; granules blue. Harden at least a week in alcohol.

1. Stain in the following solution twenty-four hours:

Alum-carmine solution,	200;
Saturated solution of dahlia in absolute alcohol,	200;
Glycerin,	100;
Glacial acetic acid,	20.

(Stir repeatedly, then allow the mixture to stand for some time.)

2. Decolorize for twenty-four hours in absolute alcohol.
3. Xylol, xylol balsam.

Unna's Isolated Stains for Mast-cells.—Harden in alcohol. Nuclei blue; cytoplasmic granules of mast-cells red.

A.—1. Stain in polychrome methylene-blue solution, plus a little alum, for three hours to over-night.

2. Wash in water.
3. Absolute alcohol, xylol, xylol balsam.

B.—1. Stain in polychrome methylene-blue solution one-quarter of an hour.

2. Wash in water.

3. Decolorize in glycerin-ether mixture for five to ten minutes.

4. Wash a long time in water.

5. Absolute alcohol, xylol, xylol balsam.

PLASMA-CELLS.

Plasma-cells arise from lymphocytes. They are often abundant in subacute and chronic pathological processes, and are characterized by cytoplasm which stains quite deeply in alkaline methylene-blue solutions. The eosin-methylene-blue stain after fixation in Zenker's fluid brings them out very sharply. The two methods best suited for their demonstration furnish at the same time a differential color-stain for mast-cells. The granules of the latter are stained red, the plasma-cells are stained blue.

Unna's Differential Stains for Plasma-cells and Mast-cells.—Harden tissues in absolute alcohol.

A.—1. Stain paraffin sections in polychrome methylene-blue one-quarter of an hour to over-night.

2. Decolorize in a small dish of water, to which are added a few drops of glycerin-ether mixture.

3. Wash thoroughly in water.

4. Absolute alcohol, xylol, balsam.

B.—1. Stain in polychrome methylene-blue solution five to fifteen minutes.

2. Wash in water.

3. Decolorize and dehydrate in a $\frac{1}{4}$ per cent. alcoholic solution of neutral orcein (about fifteen minutes).

4. Absolute alcohol, xylol, balsam.

Methyl-green-Pyronin Stain (Unna-Pappenheim).

Methyl-green,	0.15;
Pyronin,	0.25;
Alcohol,	2.50;
Glycerine,	20.00;
0.5 per cent. carbol-water to	100.00.

Fix in alcohol, stain five to ten minutes in incubator, wash in cold water, differentiate and dehydrate quickly in absolute alcohol, clear in xylol, and mount in xylol balsam. Acetone can be used to advantage in place of alcohol to dehydrate, because it has less tendency to extract the pyronin from the cytoplasm of the cells.

Schridde's Method for Demonstrating Granules in the Cytoplasm of Plasma-cells and Lymphocytes.

1. Fixation of thin slices of perfectly fresh tissue from operations in Orth's fluid warmed to 35° C. and kept at that temperature, 24 hrs.
2. Müller's fluid at room temperature, 24 to 48 "
3. Running water, 24 "
4. Distilled water, 6 "
5. One per cent. aqueous solution of osmic acid in the dark, 24 "
6. Running water, 12 "
7. Graded alcohols, } in the dark.
8. Chloroform, }
9. Imbed in paraffin.

1. Stain thin sections attached to the slide by albumen fixative in Altmann's aniline acid fuchsin solution, 2 to 24 hrs.

Acid fuchsin,	20 g.
Aniline water,	100 c.c.

2. Drain off stain and differentiate in the following solution:

Sat. alc. sol. of picric acid,	1 part;
Twenty per cent. alcohol,	7 parts

until the section acquires a clear yellowish-red color.

3. Dehydrate in alcohol.
4. Xylol.
5. Xylol balsam.

The neutrophilic granules are stained red; the acidophilic, blue.

THE COLLAGEN FIBRILS AND RETICULUM OF CONNECTIVE TISSUE.

Several methods are available for the demonstration of collagen fibrils and reticulum. The simplest is by means of Van Gieson's picric acid and acid-fuchsin solution, but it is applicable to the coarser fibers only. The stain with aniline blue is believed to be better than any yet proposed, but is limited to tissues hardened in Zenker's fluid.

A. **Mallory's Aniline Blue Stain.**—The following method is not absolutely differential because, besides collagen fibrils and reticulum, it also stains certain hyaline substances, but these latter usually are so different morphologically that confusion cannot arise. The method is also useful for the study of fibrin, fibroglia fibrils, smooth and striated muscle-fibers, and amyloid.

1. Fix in Zenker's fluid.
2. Imbed in celloidin or paraffin.
3. Stain sections in a 0.5 per cent. aqueous solution of acid fuchsin for five minutes or longer, depending on the freshness of the tissue.
4. Transfer directly to the following solution and stain from ten to twenty minutes or longer:

Aniline blue soluble in water (Grübler),	0 5 ;
Orange G (Grübler),	2.0 ;
One per cent. aqueous solution of phosphomolybdic acid,	100.00.

5. Wash and dehydrate in several changes of 95 per cent. alcohol.
6. Clear in xylol.
7. Xylol balsam.

For celloidin sections use 95 per cent. alcohol and clear by the xylol blotting-paper method.

The collagen fibrils and reticulum of connective tissue, amyloid, mucus, and certain other hyaline substances stain blue; nuclei, cytoplasm, fibroglia fibrils, axis-cylinders, neu-

roglia-fibers, and fibrin red; red blood-corpuscles and myelin-sheaths yellow; elastic fibers pale pink or yellow. The various structures do not stain with equal intensity, so that certain ones are brought out with great sharpness. This is particularly true of the collagen fibrils and reticulum of connective tissue, and of fibrin and smooth and striated muscle-fibers.

If it is desired to bring out the collagen fibrils as sharply as possible, omit the staining with acid-fuchsin. Then the nuclei and protoplasm stain yellow, and the blue fibrillæ and reticulum stand out more prominently.

B. **Van Gieson's Stain.**—The proportions given are those recommended by Freeborn. Occasionally it will be found necessary to increase the proportion of the acid fuchsin.

1. Harden in chrome salts or in corrosive sublimate. The results after alcohol are not so good.
2. Stain deeply in alum-hematoxylin.
3. Wash in water.
4. Stain for three to five minutes in

1 per cent. aqueous solution of acid fuchsin,	5 c.c.
Saturated aqueous solution of picric acid,	100 "

5. Dehydrate in 95 per cent. alcohol.
6. Oleum origani cretici.
7. Xylol balsam.

C. **Unna's Orcein Stain.**—1. Harden in alcohol.

2. Stain in the concentrated solution of polychrome-methylene-blue five minutes.
3. Wash in water.
4. Decolorize, differentiate, and stain in a 1 per cent. solution of orcein in absolute alcohol fifteen minutes.
5. Wash in absolute alcohol.
6. Xylol.
7. Balsam.

Nuclei, dark blue; cytoplasm, pale blue; elastic and connective-tissue fibers, deep orcein red; smooth muscle-fibers, bluish; mast-cell granules, red; cytoplasm of plasma-cells, deep blue.

D. **Bielschowsky's Silver Stain.**—His method for staining collagen fibrils is the same as for axis cylinders, except that step 7, the treatment with acetic acid, is omitted. The collagen fibrils are stained intensely black on a clear background. The method is highly recommended by Herxheimer.

E. **Mall's Differential Method for Reticulum.**—1. Digest frozen sections of fresh tissue, 40 to 80 μ thick, for twenty-four hours in the following solution:

Parke, Davis & Co.'s pancreatin,	5 grams;
Bicarbonate of sodium,	10 "
Water,	100 c.c.

2. Wash carefully in clean water.
3. Place sections in a test-tube half full of water, and shake thoroughly in order to remove all the cellular débris.
4. Spread out on slide, and allow to dry.
5. Allow a few drops of the following solution to dry on surface:

Picric acid,	10 grams;
Absolute alcohol,	33 c.c.;
Water,	300 "

6. Stain for about half an hour in the following solution:

Acid fuchsin,	10 grams;
Absolute alcohol,	33 c.c.;
Water,	66 "

7. Wash in the picric acid solution for a moment
8. Alcohol, xylol, balsam.

FIBROGLIA FIBRILS.

Connective-tissue cells or fibroblasts are characterized by the production of two kinds of fibrils, fibroglia fibrils, which bear the same relation to the connective-tissue cells that neuroglia fibrils bear to glia cells, and collagen fibrils, which are independent of the cells and occur between them. Fibroglia fibrils can be studied to best advantage in actively grow-

ing connective tissue, for example, in chronic salpingitis and in the stroma of carcinomata; but they are found well developed in other situations also, as, for example, in the capsules of Pacinian corpuscles.

Fibroglia fibrils can be stained differentially by several different methods. The two simplest and most useful are phosphotungstic-acid hematoxylin and the aniline blue stain after fixation in Zenker's fluid. The fibrils are often stained intensely with eosin, in eosin methylene-blue preparations, if the tissue was perfectly fresh when fixed.

A. **Mallory's Phosphotungstic-acid Hematoxylin Stain.** —Follow the directions given for neuroglia fibrils on page 369.

B. **Mallory's Aniline Blue Stain.**—(See directions on page 338.)

C. **Mallory's Acid-fuchsin Stain.** — 1. Fix in Zenker's fluid. The tissue should be as fresh as possible, and cut into thin sections (2 to 4 mm. thick) for the best results.

2. Stain celloidin or paraffin sections in a 1 per cent. aqueous solution of acid fuchsin overnight in the cold, or twenty to thirty minutes in the paraffin oven (56° C.).

3. Wash quickly in water (not over five seconds). Water extracts acid fuchsin very rapidly.

4. Differentiate in a 0.25 per cent. aqueous solution of permanganate of potassium for twenty to forty seconds. This step must not be prolonged beyond the exact time needed or the section will be decolorized.

5. Wash quickly in water (not over five seconds).

6. Dehydrate in alcohol.

7. Clear in xylol.

8. Mount in xylol balsam.

While not an absolutely differential stain for these fibrils, the method, for the most part at least, is perfectly satisfactory. It stains intensely red, not only these fibrils and the cell nuclei, but also fibrin, the contractile elements of striated muscle-cells, the differentially staining fibrils of smooth muscle-cells, neuroglia fibers, and the cuticular surfaces of epi-

thelial cells. The collagen fibrils of connective-tissue cells appear from brownish-yellow to colorless; elastic fibrils, unless degenerated, are bright yellow.

ELASTIC FIBERS.

Elastic fibers are not affected by dilute caustic soda or potash, or by acids. These reagents are often used, therefore, to demonstrate elastic fibers in the fresh condition, as, for example, in sputum, because they render them prominent by clearing or destroying the other tissues. The fibers show a marked affinity for osmic acid, staining with greater rapidity than most other tissue-elements.

For bringing out elastic fibers in sections of hardened tissues there are three excellent differential stains. The great advantage of Verhoeff's is that it is applicable after Zenker fixation.

A. **Weigert's Stain for Elastic Fibers.**— Fixation in alcohol or formaldehyde is preferable, but other fixing reagents give good results. Imbed in celloidin or paraffin. After fixation in Zenker's fluid, sections stain slowly, and there is a greater tendency, perhaps, to diffuse coloring of the collagen fibrils.

1. Stain sections twenty minutes to one hour in the following solution:

Fuchsin,	2;
Resorcin,	4;
Water,	200.

Boil the solution in a porcelain dish; when briskly boiling add 25 c.c. of liquor ferri sesquichlorati; stir and boil for two to five minutes. A precipitate forms. Cool and filter. The filtrate is thrown away. The precipitate remains on the filter-paper until all the water has drained away or until the precipitate has thoroughly dried. Then return filter and precipitate to the porcelain dish, which should be dry, but which should contain whatever part of the precipitate remained sticking to it. Add 200 c.c. of 95 per cent.

alcohol, and boil. Stir constantly, and fish out the filter-paper as the precipitate is dissolved off. Cool; filter; add alcohol to make up the 200 c.c. Add 4 c.c. of hydrochloric acid.

2. Wash off in alcohol.

3. Blot with filter-paper, and add xylol quickly; repeat the blotting, followed by xylol, two or three times until the section is perfectly cleared.

4. Xylol balsam.

Sections can be stained for several hours. If the rest of the tissue is overstained, differentiate in acid alcohol; if the sections are too deeply stained, the color cannot be washed out. Diffuse staining can be avoided by diluting the stain either with alcohol or, better still, with alcohol containing 2 per cent. of hydrochloric acid. The elastic fibers appear dark blue, almost black, on a clear background. The nuclei can be stained red with carmine before or after the staining of the fibers. After Zenker fixation, carmine stains are difficult to obtain. A light nuclear stain with alum-hematoxylin, after the fibrils have been colored, is preferable.

The solution keeps for months.

If it be desired to keep sections for some time before mounting, wash them in alcohol and place in water.

B. **Unna's Orcein Method for Elastic Fibers.**—Unna's latest method of using orcein is as follows, and can be highly recommended:

1. Stain sections in the following solution:

Orcein (Grübler),	1;
Hydrochloric acid,	1;
Absolute alcohol,	100.

Place the sections in a dish and pour over them enough of the solution to cover them. Warm gently in an incubator or over a small flame for ten to fifteen minutes until the solution thickens, or leave in the solution at room-temperature overnight.

2. Wash off thoroughly in dilute alcohol (70 per cent.).

3. Wash in water to get rid of all the acid and to fix the color.

4. Alcohol.
5. Oil.
6. Balsam.

The washing in water is not absolutely essential.

Elastic fibers are stained of a deep silky-brown color, connective tissue a pale brown. If it is desirable to have only the elastic fibers stained, wash for a few seconds in 1 per cent. hydrochloric-acid alcohol before washing in water. The nuclei can be brought out by staining in Unna's polychrome methylene-blue solution after washing the sections in water.

Verhoeff's Elastic Tissue Stain.—Fixation in formaline or Zenker's fluid preferred. Tissues or sections should not be treated with iodine solution before staining. Mercurial precipitates, if removable, are removed by the staining solution. For the best results the solution should be used within twenty-four hours, but satisfactory specimens may be obtained with solutions one month old.

The staining fluid is made as follows:

Hematoxylin crystals,	1 gm.
Absolute alcohol,	20 c.c.

Dissolve in test-tube by aid of heat, filter, and add in order given:

Aqueous solution (10 per cent.) of ferric chlorid,	8 c.c.
Lugol's solution (iodin, 2; potassium iodid, 4; water, 100),	8 c.c.

Sections are immersed in the staining fluid for fifteen minutes or longer until perfectly black, and are then differentiated in a 2 per cent. aqueous solution of ferric chlorid. The differentiation requires only a few seconds. To observe the stages in the differentiation, the sections may be examined in water under a low magnification. If the differentiation has been carried too far, the sections may be restrained, provided that they have not been treated with alcohol.

The sections are now washed in water, followed by 95 per cent. alcohol to remove the stain of the Lugol solution, and

then are allowed to remain in water five minutes or longer. They are then counterstained in a ½ per cent. aqueous solution of eosin if desired, passed through alcohol, oil of origanum, and mounted in balsam.

By this method elastic tissue is stained black, while connective tissue, fibroglia, myoglia, and neuroglia fibrils, myelin, and fibrin take the eosin stain. Nuclear staining may be obviated by doubling the amount of Lugol's solution in the staining fluid. Degenerated elastic tissue (elacin) is also stained by this method. The degenerated fibrils may be distinguished from the normal by staining less intensely and presenting less distinct outlines.

Equally good results, especially after Zenker's fixation, may be obtained by staining the tissues *en masse*. Myelin, however, is also stained. Thin slices of tissue after fixation are removed from 80 per cent. alcohol and immersed in the staining fluid four days. They are then quickly rinsed in water to remove excess of stain, placed in 80 per cent. alcohol, and imbedded in the usual manner. The sections are differentiated in a ½ per cent. solution of ferric chlorid.

SMOOTH AND STRIATED MUSCLE-CELLS.

Smooth and Striated Muscle-cells.—For the demonstration of muscle-cells double stains, such as alum-hematoxylin and eosin or eosin and methylene-blue, are sufficient.

For bringing out the finer details in the cytoplasm, however, phosphotungstic-acid hematoxylin and the aniline blue stain are much to be preferred. It is imperative that the tissue be perfectly fresh, especially if the myoglia fibrils in smooth muscle-cells are to be studied, because they very quickly undergo postmortem changes. Thin sections of the tissues to be studied should be put into Zenker's fluid within five to ten minutes at the most after removal from the body, if the best results are desired. Autopsy material is practically useless. The most desirable tissues are those obtained directly at operations on the human body.

A. Phosphotungstic-acid Hematoxylin Stain (Mallory). For directions see page 369.

B. Aniline Blue Stain (Mallory). (See page 338.)

C. **Benda's Stain for Myoglia Fibrils.**—1. Fix fresh material in Zenker's fluid for twenty-four hours.

2. Wash for a number of hours in water.

3. Make frozen sections.

4. Place sections in a 0.5 per cent. solution of chromic acid for twenty-four hours.

5. Wash off in water.

6. Place in a 0.25 per cent. solution of permanganate of potassium for about three minutes.

7. Wash off in water.

8. Place in Pal's mixture of sulphite of sodium and oxalic acid for five minutes.

9. Wash off in water; take up section on slide.

10. Cover with the following solution:

Crystal-violet, saturated solution in 70 per cent. alcohol,	1 part;
Acid alcohol,	1 "
Aniline-water,	2 parts.

11. Blot with filter-paper.

12. Cover with dilute Lugol's solution.

13. Blot with filter-paper; dry.

14. Differentiate in aniline oil and xylol, equal parts.

15. Xylol; xylol balsam.

THE CENTRAL NERVOUS SYSTEM.

In the preservation of the central nervous system the special structures which require consideration are the ganglion-cells, including both the dendritic and the axis-cylinder processes, the myelin-sheaths, and the neuroglia-fibers. No one fixing reagent is suited for the best preservation of all of them, unless possibly it be formaldehyde.

The main fixing fluids for the nervous system until within a very short time have been various solutions of the chrome salts, particularly of bichromate of potassium, either alone or in combination with sulphate of sodium, as in the well-

known Müller's fluid. The chief objections to the chrome salts as fixatives are that they penetrate and harden very slowly, and do not preserve properly either the ganglion-cells or the neuroglia-fibers. On the other hand, they probably preserve the axis-cylinders as well as any reagent we yet know, and are invaluable for their property of entering into some chemical combination with myelin, in consequence of which it is possible to obtain by the method originated by Weigert a differential stain of the myelin-sheaths.

The new fixing reagent, formaldehyde, seems likely to find its greatest use histologically as a fixative of the central nervous system. It penetrates and hardens up to a certain degree with great rapidity. It also preserves in certain structures the special chemical properties on which certain differential stains depend. Small pieces of nervous tissue are properly fixed in the standard solution (4 per cent. solution of formaldehyde gas) in four days. A whole brain will be so hardened in ten days to two weeks that thin serial sections can be made through it without fear of the slices altering their shape in the least. The process could undoubtedly be hastened by injecting the arteries.

It must be borne in mind, however, that for most purposes formaldehyde must be followed by other reagents before the structures and their chemical properties preserved by it are properly fixed so that they will not be altered when transferred to alcohol. In other words, formaldehyde may be looked upon as a very quick preliminary fixing reagent. The hardening of brains entire in it is not recommended, except in certain cases—for instance, of cysts, hemorrhages, or occasionally of tumors—where the gross lesions and the tracts or structures affected by them are of more importance than the finer histological changes. For the proper preservation of ganglion-cells and of neuroglia-fibers very small pieces must be taken and fixed by the special methods given; but if the main object is to trace system-degenerations, much larger pieces, or even the whole brain, may be taken, because the myelin-sheaths change comparatively very slowly after death.

The stains for the central nervous system may be divided into two classes—general and differential. For nearly all of them preliminary fixation in formaldehyde is advisable or possible. This renders the immediate preservation of nervous tissue very simple, and at the same time allows a variety of mordanting and staining methods to be used later.

The staining of the various histological elements of the central nervous system and the fixing reagents best suited for each of them will be considered under the following headings:

General Stains.

Stains for ganglion-cells, { Cytoplasmic granules; Dendritic and axis-cylinder processes; Axis-cylinders and their terminal processes.

Stains for the myelin-sheath.

Stains for the neuroglia-fibers.

General Stains.—General stains include the ordinary nuclear stains, with or without a contrast-stain, and certain diffuse single or combined stains which color the nuclei, the cell-protoplasm, including to a varying extent the dendritic processes of the ganglion-cells, the axis-cylinders, and the neuroglia-fibers. The different stains vary somewhat in regard to the structures which they bring out most prominently.

The best fixation for the general stains is Zenker's fluid, to be followed by the eosin-methylene blue and the phosphotungstic acid hematoxylin stains. Alum-hematoxylin, followed by eosin, is sometimes useful. The eosin, if deep enough, brings out fairly well both the dendrites and the axis-cylinders.

The various carmine solutions, particularly neutral, ammonia, and picro-carmine, have long been the favorite diffuse stains for the central nervous system, but the uncertainty of their action and the difficulty of always getting a good staining solution have gradually led to the introduction of more reliable methods. Of these, the simplest, quickest, and in many ways the most generally useful is—

A. **Van Gieson's Stain.**—It may be used after any fixation. Although this mixture of acid fuchsin and picric acid

may be made up in the way originally recommended, the following exact proportions, given by Freeborn for staining nervous tissues, will be found generally preferable:

1 per cent. aqueous solution of acid fuchsin,	15 c.c.;
Saturated aqueous solution of picric acid,	50 "
Water,	50 "

1. Stain sections first rather deeply in alum-hematoxylin.
2. Wash in water.
3. Stain in above solution three to five minutes.
4. Dehydrate in alcohol.
5. Oil, xylol balsam.

The nuclei appear bluish red, the ganglion-cells and processes red, the axis-cylinders brownish-red, the myelin-sheaths yellow, the neuroglia-fibers orange red, connective-tissue fibrillæ deep red. After certain reagents this solution will not give a sufficiently intense stain. In such cases a mixture of 1 part of a 1 per cent. solution of acid fuchsin to 2 parts of a saturated solution of picric acid is recommended.

B. **Phosphotungstic-acid hematoxylin** (see page 295) will be found of much value as a general stain for the central nervous system if employed in the manner recommended for neuroglia-fibers after fixation by the method there given, because a greater differentiation of the various tissue-elements is obtained than by any other method.

C. **Phosphomolybdic-acid Hematoxylin** (see page 295).—This solution stains well only after fixation in a simple chrome salt, as in Müller's fluid.

1. Stain sections twenty minutes to one hour.
2. Wash out in two or three changes of 50 per cent. alcohol until the celloidin becomes completely decolorized (about five minutes).
3. Dehydrate in 95 per cent. alcohol.
4. Oil, xylol balsam.

The ganglion-cells are often overstained, especially if the tissue has been hardened but recently. The method is particularly good for bringing out the axis-cylinders and the neuroglia-fibers.

D. **Aniline Blue Stain** (see page 297).—The method recommended for connective-tissue fibers will also be found very useful for the study of the nervous system. The best results are obtained after fixation in Zenker's fluid.

E. **Nigrosin.**—1. Stain sections in a concentrated aqueous solution of nigrosin five to ten minutes.

2. Decolorize and dehydrate in weak, then in strong, alcohol.

3. Oil, Canada balsam.

The stain is not very sharp, but is simple and useful, particularly for low-power observation.

Stains for Nissl or Tigroid Bodies.—These bodies are brought out with great sharpness by the eosin-methylene blue stain after fixation in Zenker's fluid, but the following are the classical methods for demonstrating them: *A.* **Nissl's Stain.**—1. Carefully harden pieces of tissue not over 1 to 1.2 cm. in diameter in 96 per cent. alcohol.

2. Cut sections without imbedding, as follows: Remove excess of alcohol from tissue with filter-paper; dip base of specimen in thick celloidin; mount on block; harden in 96 per cent. alcohol. Moisten knife with 96 per cent. alcohol. Sections should always be under $\frac{1}{100}$ mm. in thickness. In order to be able to compare the number of cells, etc., in one case with those in another, the sections should be of uniform thickness. Preserve sections in 96 per cent. alcohol.

3. Stain the sections in the following solution heated over a flame until it bubbles noisily (60°–70° C.):

Methylene-blue, B patent,[1]	3.75;
Venetian soap,	1.75;
Distilled water,	1000.

4. Wash out in—

Aniline oil,	10 parts;
96 per cent. alcohol,	90 "

until the color is no longer given off in coarse clouds.

[1] Nissl personally prefers and uses the make of Carl Buchner und Sohn, Munich.

5. Transfer section to slide; dry with filter-paper and cover with oil of cajuput.

6. Blot with filter-paper, and then wash with a few drops of benzine.

7. Add a few drops of benzine-colophonium (made by dissolving colophonium in benzine for twenty-four hours and then decanting).

8. Hold the slide above the flame until all the benzine is driven off. (Nissl no longer recommends burning off the benzine.)

9. Cover-slip. Warm the slide, so that the colophonium will spread out evenly between the cover-slip and the slide.

The specimen is now mounted in a medium in which diffusion of color cannot take place, so that the stain is practically permanent. The best results are obtained with tissues which have not been hardened in alcohol over one to four days. Contact with water, weak alcohol, and ether must be avoided.

B. **Lenhossek's Stain.**—The following method will be found simpler, but the specimens are not permanent.

1. Harden sections in 90 per cent. alcohol, then in 96 per cent., or in formaldehyde followed by alcohol. Do not keep the tissues too long in alcohol.

2. Imbed sections in celloidin or paraffin, or cut without imbedding, as in Nissl's method.

3. Stain sections in a completely saturated solution of thionin for five minutes.

4. Wash for a few seconds in water.

5. Differentiate in aniline, 1 part;
Absolute alcohol, 9 parts.
Do not decolorize too long.

6. Clear in oleum cajuputi.

7. Xylol.

8. Xylol balsam.

The granulations can be shown by other stains, such as safranin, fuchsin, dahlia, alum-hematoxylin.

Ganglion-cells; Dendritic and Axis-cylinder Processes.—**Golgi's Methods.**—Golgi's methods, although of

the greatest value in the study of the normal histology of the central nervous system, are of very little use in the study of its pathology. The reason of this is the very peculiarity that makes the method of value in normal histology—namely, that it picks out here and there a cell and stains it with all its wealth of processes more or less completely, while the neighboring cells are left colorless. If all of the cells and their processes were stained, the picture presented would be a confused mass. In pathological histology, where the presence or absence of certain cells or processes is of paramount importance, it is of primary necessity that every cell within a given area shall be perfectly stained.

Golgi introduced three different methods of obtaining the stain now called after his name. They are spoken of as the slow, the mixed, and the short methods. Golgi himself employed principally the first two methods, and they are still used for the study of the developed brain and cord.

The quick method exclusively has been used by Ramon y Cajal and other recent investigators for the study of embryonic nervous tissue.

The following points are to be borne in mind: The tissue should be as fresh as possible, and should be cut into small pieces, not over 1 to 1½ cm. thick—for the quick method even thinner. With the corrosive-sublimate method, however, larger pieces can be used.

Large quantities of the solutions should be used—at least ten times the volume of the specimen It is best to keep the specimens in the solution in the dark, especially in using the corrosive-sublimate method.

Golgi's Slow Method.—1. Harden the tissues in a 2 per cent. solution of bichromate of potassium two to six weeks. In summer fifteen to twenty days are sufficient; in winter, unless the temperature is kept at 25° C., one to one and a half months will be required. Keep the specimens in the dark. Large amounts of the solution should be used, and it should be frequently changed, especially during the first week.

2. Transfer either to (*a*) a ¾ per cent. solution of nitrate

of silver for twenty-four to forty-eight hours; a longer time will do no harm; or to (*b*) a $\frac{1}{2}$ per cent. solution of corrosive sublimate—small pieces eight to ten days, large pieces two months or more. Change the solution frequently during the first few days; later only when the solution gets yellow.

This second procedure is recommended for larger pieces of tissue than can properly be impregnated by (*a*).

Golgi's Mixed Method.—1. Harden small pieces of tissue for three to five days or longer in a 2 per cent. solution of bichromate of potassium at 25° C., in the dark.

2. Transfix to a mixture of—

1 per cent. solution of osmic acid,	2 parts;
2 per cent. solution of bichromate of potassium,	8 "

for three to eight days.

3. Place in a $\frac{3}{4}$ per cent. solution of nitrate of silver for twenty-four to forty-eight hours.

Golgi's Quick Method.—1. Small pieces of fresh tissue are placed directly in the following solution:

1 per cent. solution of osmic acid,	1 part;
3.5 per cent. solution of bichromate of potassium,	4 parts,

for several days (three to eight).

2. They are then transferred to a large amount of a $\frac{3}{4}$ per cent. solution of nitrate of silver for one, two, or six days.

The length of time the tissues should remain in the osmic-acid and bichromate-of-potassium solution depends on what elements it is desired to impregnate. In the human cord the time is in general the following:

1. Neuroglia,	2–3 days;
2. Nerve-cells,	3–5 "
3. Nerve-fibers and collaterals,	5–7 "

The further treatment of the tissues impregnated by these methods is as follows: Alcohol must be avoided as much as possible. The tissues are usually firm enough to cut after the impregnation; if not, place in absolute alcohol for fifteen to thirty minutes. The sections should be rather thick, $\frac{1}{20}$

to $\frac{1}{10}$ mm. They may be made free hand with a razor or in the microtome. For either method the tissues can be held between pieces of elder-pith, or may be quickly imbedded in celloidin by dehydrating for a few minutes in absolute alcohol and then placing in a thick solution for five minutes. From the celloidin they are mounted in elder-pith or on blocks, and placed for a short time in 80 per cent. alcohol to harden.

Treatment of Sections.—1. Dehydrate quickly in alcohol.

2. Clear in oil of cloves or bergamot.

3. Wash off with xylol.

4. Mount without a cover-glass in xylol damar, and dry quickly at 40° C.

The mounted sections must be protected from the light and from dust as much as possible. Cajal has modified Golgi's quick method by repeating the steps (Cajal's so-called double method) so as to get a more perfect impregnation. The same osmic-acid and bichromate-of-potassium solution may be used over again, or a fresh solution, containing about one-half as much osmic acid, is made up fresh. The silver solution should be taken fresh each time. Lenhossek, Weigert, and others have obtained very good Golgi preparations with tissues first fixed in formaldehyde.

Of the various methods proposed for fixing the Golgi stains so that contrast-stains could be used with them and the specimens protected by cover-slips, the simplest and most practical seems to be that advocated by Kallius.

The Method of Kallius for Fixing Golgi Stains.—The method depends on the employment of a photographic developer to reduce the bichromate of silver to metallic silver.

1. Place sections for several minutes in a solution composed of 1 part of the following developer:

Hydrochinon,	1;
Sulphite of sodium,	8;
Carbonate of potassium,	1.5;
Water,	575,

plus one-third to one-half as much absolute alcohol until the sections become gray to black in color. If too much alcohol is added, the carbonate of potassium will be precipitated, but will redissolve on the addition of a little more developer.

2. 70 per cent. alcohol for ten to fifteen minutes.

3. Hyposulphite of sodium (20 per cent. aqueous solution).

4. Wash thoroughly in a large amount of water for twenty-four hours.

5. Alcohol, oil, xylol balsam; cover-glass.

Cox's Modification of Golgi's Corrosive-sublimate Method.—The same black pictures are obtained by this method as by Golgi's, but with this difference, that nearly all of the cells in the section are impregnated. This is an advantage when the topographical arrangement of the cell-layers is desired, but a disadvantage when it comes to the study of individual cells, because on account of the luxuriance of the impregnation such a study is rendered impossible. Small pieces of nervous tissue are placed in the following solution:

Bichromate-of-potassium 5 per cent. solution,	20;
Corrosive-sublimate 5 per cent. solution,	20;
Distilled water,	30–40;
Simple chromate-of-potassium 5 per cent. solution,	16.

The time required for impregnation is a month in summer and two to three months in winter. The after-treatment is the same as for Golgi preparations.

Axis-cylinders and their Terminal Processes.—The three methods most in use in the past for the study of central and peripheral nerve-fibers and their terminations are the gold, the Golgi, and the methylene-blue methods. All three may give beautiful results, but, as a rule, they are very unreliable. Their use is confined almost wholly to the study of normal tissues. More recently, Bielschowsky's silver impregnation method has come to the front and has been found of considerable value in pathological work.

1. **Gold Stain for Nerve-fibers.**—For the application of the gold method to fresh tissues see p. 315.

Various attempts have been made to devise a reliable method of employing chlorid of gold for staining nerve-fibers in sections of hardened tissues. The results have not been altogether successful. The best results can probably be obtained by—

A. Gerlach's Method.—1. Harden tissues in a 1–2 per cent. solution of bichromate of ammonium for one to three weeks; cut sections without passing through alcohol, which must be avoided.

2. Place the sections in a very dilute solution ($\frac{1}{100}$ per cent.) of the double chlorid of gold and potassium very slightly acidulated with hydrochloric acid, for ten to twelve hours, until they become slightly violet in color.

3. Wash in a solution of hydrochloric acid 1, to water 2000–3000.

4. Place for ten minutes in a $\frac{1}{10}$ per cent. solution of hydrochloric acid in 60 per cent. alcohol.

5. Absolute alcohol, oil of cloves, Canada balsam.

Another method frequently recommended is the following:

B. Freud's Gold Stain for Nerve-fibers.—1. Harden tissues in Erlicki's or Müller's fluid, followed by alcohol. Imbed in celloidin.

2. Stain sections three to five hours in 1 per cent. solution of chlorid of gold, and 95 per cent. alcohol, equal parts.

3. Wash in water.

4. Reduce in—

Caustic soda,	1;
Distilled water,	6,

for two to three minutes.

5. Wash in water.

6. Place for five to fifteen minutes in a 10 per cent. solution of iodid of potassium.

7. Wash in water.

8. Alcohol, oil, xylol balsam.

2. **Methylene-blue Stain for Nerve-fibers.**—The methylene-blue method is due to Ehrlich. Many modifications of the original procedure have been suggested with a view to making the results surer or the specimens more permanent. Tissues can be stained either by injection or by immersion.

The methylene-blue used should be Grübler's "rectified methylene-blue for vital injection."

For injection in the blood- or lymph-vessels of live or dead animals a 1 to 4 per cent. solution in normal salt solution is recommended. The injected organs are exposed to the air until a bluish tint is visible. As soon as the greatest intensity of stain is reached (five minutes to two hours) the color in the preparation is fixed by placing small bits of the tissue in a freshly-filtered, cold, saturated, aqueous solution of picrate of ammonium, or, better still, in the solution given below, recommended by Bethe.

Very small or thin pieces of tissue intended for staining by immersion (the method employed for human tissues) are placed in a very dilute solution ($\frac{1}{16}$–$\frac{1}{15}$ per cent.) of methylene-blue in normal salt solution. Lavdowski recommends very highly a solution of methylene-blue in egg-albumin, either alone or combined with chlorid of sodium or ammonium. The white of egg is freed from the thicker portions or filtered. When the experiment is to last some time, add to the egg-albumin an equal part of a $\frac{1}{2}$ per cent. solution of chlorid of sodium or of a $\frac{1}{4}$ per cent. solution of chlorid of ammonium. The tissue, protected by a large dish, is exposed to the air for fifteen minutes to twelve hours, until the maximum stain is obtained.

The stain may then be fixed by the method already given, or, better still, in the following manner:

Bethe's Method of Fixing Methylene-blue Stains of Nerve-fibers.—1. Wash off excess of color with normal salt solution.

2. Place in—

Molybdate of ammonium,	1 gr.;
Distilled water,	10 c.c.;
Peroxid of hydrogen,	1 "
Hydrochloric acid,	1 drop.

A precipitate forms on making up the solution, but disappears on shaking. The solution will keep eight days, but is best made up fresh each time. It should be used as cold as possible, preferably surrounded by a mixture of ice and salt. Leave the tissue in the cold solution for from two to five hours, and then for a while longer at the room-temperature.

3. Wash one half to two hours in running water.

4. Dehydrate and harden as quickly as possible (not over twelve to twenty-four hours) in cold absolute alcohol. (The color is soluble in warm alcohol.)

5. Clear in xylol.

6. Imbed in paraffin.

The sections may be mounted directly or brought into water and stained with alum-cochineal for contrast. If a little osmic acid be added to the fixing solution after the specimens have been in it for a while, a more permanent methylene-blue stain is obtained.

3. *Bielschowsky's method of staining axis-cylinders and neurofibrils by silver impregnation:*

1. Fix in 4 per cent. formaldehyde (10 per cent. formol) for twenty-four hours or longer.

2. Cut very thin sections (5 μ) with the freezing microtome and place them in distilled water. (Bielschowsky has recently advised transferring the sections from the water to pure pyridin for twenty-four to forty-eight hours and then washing thoroughly in distilled water before proceeding with step 3).

3. Transfer them to a 2 to 3 per cent. solution of nitrate of silver in distilled water for twenty-four, preferably forty-eight, hours.

4. Wash quickly in distilled water.

5. Place the sections in the following solution for two to three minutes (as a rule, about five minutes):

To 10 c.c. of a 10 per cent. aqueous solution of nitrate of silver add 5 drops of a pure 40 per cent. aqueous solution of sodium hydrate. A precipitate is formed, which is to be dissolved by adding ammonia drop by drop while stirring constantly with a glass rod. The ammonia must not be added in excess, but only enough to dissolve the precipitate. It is

best to leave a trace of precipitate and filter. Add to the filtrate enough distilled water to make the solution measure 20 c.c. This solution contains the easily reducible silver salts, ammonium nitrate of silver, and ammonium oxid of silver.

6. Wash in distilled water.

7. Place in weak acetic acid (5 drops of glaciāl acetic acid to 20 c.c. of distilled water) until the color of the sections changes from brown to dark yellow.

8. Transfer the sections to an 8 per cent. solution of formaldehyde (20 per cent. formol) for five to thirty minutes until no more white clouds are given off. In this solution reduction to metallic silver occurs. The sections appear brownish to black, depending on how long they were washed in the distilled water.

The sections are now toned with gold in order to intensify the stain of the fibrils and to render it more permanent.

9. Wash quickly in distilled water.

10. Place sections in the following gold bath: namely, 5 drops of a 1 per cent. gold chlorid solution in 10 c.c. of distilled water (to which it is sometimes advisable to add 2 or 3 drops of glacial acetic acid), for ten minutes.

11. Removal of any unreduced silver by placing the sections in a 5 per cent. solution of hypophosphite of sodium (to which 1 to 2 drops of a saturated solution of sodium sulphite are sometimes added) for one-half to one minute.

12. Wash thoroughly in water.

13. Dehydrate quickly in absolute alcohol: xylol; balsam.

Bielschowsky has recently modified his method as follows for staining tissues in mass before cutting, but it is not so reliable as the stain for frozen sections:

1. Fix in formaldehyde.

2. Wash in water.

3. Place in pure pyridin for three to four days.

4. Wash thoroughly in distilled water.

5. Place in a 3 per cent. solution of silver nitrate for three to five days.

6. Wash quickly in water.

7. Place in the second silver bath (step 5 in directions above) diluted to 86 c.c. for twenty-four hours.

8. Wash in water.

9. Reduce in formaldehyde.

10. Dehydrate in graded alcohols; clear and imbed in paraffin; cut sections.

11. Xylol, balsam.

The axis cylinders and nerve-fibrils are stained black.

4. *Stroebe's Aniline-blue Stain for Nerve-fibers in Hardened Sections.*—Harden tissues in Müller's fluid. 1. Stain one-half to one hour in a saturated aqueous solution of aniline-blue.

2. Wash in water.

3. Transfer to a small dish of alcohol to which are added 20 to 30 drops of a 1 per cent. alcoholic solution of caustic potash (caustic potash 1 to alcohol 100: let the mixture stand for twenty-four hours; then filter). In one to several minutes the sections become bright brownish-red and transparent.

4. Transfer to distilled water for five minutes. The section becomes bright blue again.

5. Stain in a half-saturated aqueous solution of safranin, one-quarter to one-half hour long.

6. Wash out and dehydrate in absolute alcohol.

7. Xylol, xylol balsam.

5. *Chlorid-of-iron and dinitroresorcin method* for the study of degenerated peripheral nerves:

1. Place fresh pieces of peripheral nerves for several days in a solution of—

Chlorid of iron,	1 part;
Distilled water,	4 parts.

2. Wash out thoroughly in water.

3. Transfer to a saturated solution of dinitroresorcin in 75 per cent. alcohol for several weeks.

4. Wash, dehydrate, imbed, etc.

A permanent green color is formed which stains the nerves green and brings out the green axis-cylinders very sharply.

The stain will succeed with preparations which have been hardened in Flemming's solution or Müller's fluid.

Golgi's methods are sometimes employed for the study of the terminal processes of nerve-fibers (for directions see p. 351).

Stains for the Myelin-sheath.—The myelin-sheath of nerve-fibers is a form of fat, and like it possesses the property of reducing osmic acid, by means of which a selective sheath stain can be obtained. Unfortunately, however, the osmic acid penetrates to but a very slight depth. Three methods employing osmic acid are given, but they are all expensive and not so satisfactory as those employing hematoxylin.

The differential hematoxylin stain, originated by Weigert, and ordinarily used, depends on some chemical reaction which takes place between the myelin and a chrome salt, in consequence of which the myelin is fixed so that it will not later be dissolved out by alcohol or ether, and at the same time is so mordanted that it can be deeply stained with hematoxylin, to which it clings when treated with certain decolorizers. This reaction between the myelin and the chrome salts in general use takes place very slowly at the ordinary temperature; six weeks to several months are usually required. Weigert's latest method depends on the interaction of two chrome salts in the same solution, in consequence of which the time needed for this reaction or mordanting is reduced to four days. The solution may be used alone, but is best combined with formaldehyde or used after it.

A. **Weigert's Myelin-sheath Stain.**—In this method five steps are involved, but the first two can be, and often are, combined in one. These five steps are fixation, primary mordanting, secondary mordanting, staining, and differentiation. These different steps will be considered separately.

1. *Fixation.*—Place the tissues in a 4 per cent. solution of formaldehyde (10 per cent. solution of formalin) for four days to several weeks or indefinitely, using several times the volume of the tissue. Change the solution at the end of twenty-four hours, and thereafter whenever it becomes cloudy. Large masses of nervous tissue, like the medulla and pons or the basal ganglia, should be fixed in formaldehyde for one to three weeks.

2. *Primary Mordanting.*—Cut the tissues fixed in formal-

dehyde into slices not over 1 cm. thick, and place in the following solution for four to five days at room-temperature:

Bichromate of potassium,	5;
Fluorchrom,	2;
Water,	ad 100.

Steps 1 and 2 may be combined by adding 4 per cent. of formaldehyde to the mordanting solution and placing the fresh tissues directly in the mixture.

3. *Secondary Mordanting.*—Transfer the tissues to the following solution for twenty-four to forty-eight hours:

Acetate of copper,	5.0;
Acetic acid, 36 per cent. solution,	5.0;
Fluorchrom,	2.5;
Water,	ad 100.0

(For method of preparation see page 294.)

Weigert always transfers blocks of tissue to the secondary mordant, of which the function is to intensify the staining reaction. Many workers, however, prefer to employ the secondary mordant on sections only. In this case they place the tissues directly from the first mordant into graded alcohols, imbed in celloidin, and cut sections before step 3. Either way gives good results, but the first is the simpler.

Weigert also recommends the following iron solution as a secondary mordant, but if applied to blocks of tissue, they must be well washed in running water before being dehydrated and imbedded in celloidin, because otherwise the iron will rust the knife badly. Of course, if this mordant is applied to sections only, prolonged washing is not necessary:

Iron alum (ammonioferric alum),	5;
Acetic acid,	5;
Water,	ad 100.

After the secondary mordant the tissue is dehydrated in graded alcohols, imbedded in celloidin, and sections cut in the usual manner.

4. *Staining.*—Stain sections in the following solution for twelve to twenty-four hours:

Ripened 10 per cent. solution of hematoxylin in absolute alcohol,	10;
Saturated aqueous solution of carbonate of lithium,	1;
Water,	90.

Keep on hand as a stock solution a 10 per cent. solution of hematoxylin in absolute alcohol. At least ten days of exposure to light are required to ripen this solution so that it can be used for staining. Combine with the carbonate of lithium and the water at the time of using in such proportions as are wanted for immediate use. Wash the sections thoroughly in water before differentiation.

Another staining solution which Weigert recommends highly is the following—it consists of two parts:

(*a*)	Liquor ferri sesquichlorati (officinal),	4 c.c.;
	Water,	96 "
(*b*)	Ripened 10 per cent. solution of hematoxylin in absolute alcohol,	10 "
	96 per cent. alcohol,	90 "

Mix thoroughly equal parts of these two solutions just before using, and pour over the sections. Stain overnight or longer at room-temperature. Pour off solution and wash with water.

5. *Differentiation.*—The sections are differentiated in the following solution which it is sometimes advisable to dilute with water:

Borax,	2.0;
Ferricyanid of potassium,	2.5;
Water,	100.0.

After the first staining method given above the decolorized tissues appear yellow; after the iron-hematoxylin solution they are colorless. Moreover, this latter method stains the very finest fibers and at the same time the coarse fibers, such as occur, for example, in the nerve-roots.

After differentiation the sections should be thoroughly washed in water, dehydrated in alcohol, cleared in the anilin

oil-xylol or carbol-xylol mixture, and mounted in xylol balsam.

The steps of the process may be summed up as follows:

1. Fix in 10 per cent. formaldehyde four days or longer.

2. Mordant in the bichromate of potassium-fluorchrom solution four to five days.

3. Mordant in the acetate of copper-fluorchrom solution twenty-four to forty-eight hours, or in the iron solution twenty-four to forty-eight hours.

4. Dehydrate in graded alcohols.

5. Imbed in celloidin.

6. Stain sections in the alcoholic hematoxylin solution or in the iron-hematoxylin solution for twelve to twenty-four hours.

7. Wash off in water.

8. Differentiate in the borax-ferricyanid of potassium solution.

9. Wash thoroughly in water.

10. Dehydrate in alcohol.

11. Clear in the anilin oil-xylol or carbol-xylol mixture.

12. Mount in xylol balsam.

B. **Pal's Modification of Weigert's Myelin-sheath Stain.**—1. Fixation and primary mordanting as for Weigert's method.

2. Place sections for several hours in a ½ per cent. aqueous solution of chromic acid, or for a longer time in a 2–3 per cent. solution of bichromate of potassium. This step is often omitted, especially when the tissues have been but recently mordanted.

3. Transfer sections to Weigert's alcoholic hematoxylin solution for twenty-four to forty-eight hours (if necessary for an hour in the incubator at 37° C.). This solution is as follows:

Ripened 10 per cent. solution of hematoxylin in absolute alcohol,	10.
Water,	90.

4. Wash in water plus 1 to 3 per cent. of a saturated aqueous solution of carbonate of lithium until the sections appear of a uniform deep-blue color.

5. Differentiate for twenty seconds to five minutes in a $\frac{1}{4}$ per cent. aqueous solution of permanganate of potassium until the gray matter looks brownish-yellow.

6. Transfer to the following solution :

Oxalic acid,	1 ;
Sulphite of potassium,	1 ;
Water,	200.

for a few seconds until the gray matter is colorless or nearly so.

7. Wash thoroughly in water.
8. Dehydrate in 95 per cent. alcohol.
9. Oil, xylol balsam.

Steps 5 and 6 sometimes have to be repeated when the differentiation has not been complete.

Of all the numerous modifications of Weigert's original myelin-sheath stain, the only one that has found general acceptance until recently is Pal's. It has the following advantages : It gives very clear pictures ; everything except the sheaths is completely decolorized, so that contrast-stains are possible; it is more successful with thick sections than Weigert's method ; the separate steps are quicker. On the other hand, the danger of decolorizing the sheaths of the finer fibers is greater.

C. **Kulschitzky-Wolter's Modification of Weigert's Myelin-sheath Stain.**—It is claimed to stain the myelin sheaths intensely and with great certainty.

1. Fix in formaldehyde, mordant in Weigert's chromalum mixture, dehydrate in alcohol, imbed in celloidin.

2. Stain sections twenty-four hours at a temperature of 37° C in the following solution well ripened :

Hematoxylin,	1 gram ;
Absolute alcohol,	10 c.c.
2 per cent. acetic acid,	90 "

3. Dip sections in Müller's fluid.

4. Differentiate in a $\frac{1}{3}$ per cent. aqueous solution of permanganate of potassium.

5. Wash in water.

6. Place in Pal's oxalic acid solution until the gray matter is colorless.

7. Wash thoroughly in tap water to which some sal ammoniac is added.

8. Dehydrate in alcohol.

9. Clear and mount in balsam.

D. **Exner's Stain.**—The tissue should be obtained as soon as possible after death, although the method will succeed with tissues even over twelve hours old.

1. Place pieces of brain or cord not over $\frac{1}{2}$ cm. thick in a 1 per cent. aqueous solution of osmic acid, using at least ten times as much fluid as the volume of the specimen.

2. Change the osmic-acid solution on the second day.

3. After five or six days wash thoroughly in water.

4. Dehydrate, imbed, etc.

5. Examine sections in glycerin rendered slightly ammoniacal.

The myelin-sheaths appear gray to black. The preparations are not permanent.

This procedure has been almost entirely replaced by Weigert's method, which has numerous advantages. Lately, however, it has been brought forward again by Heller, who uses a photographic developer to reduce the osmic acid and to make possible permanent mounts. He has lately published the following method for sections, but it cannot be unconditionally recommended:

E. **Heller's Myelin-sheath Stain.**—1. Harden as for the Weigert method (Heller used Müller's fluid).

2. Imbed in celloidin.

3. Place sections in a 1 per cent aqueous solution of osmic acid for ten minutes in thermostat or for half an hour at room-temperature.

4. Wash in water.

5. Reduce in the following developer:

Sulphate of sodium,	125;
Carbonate of sodium,	70;
Water,	500;
Pyrogallic acid,	15.

6. Wash in water.

7. Differentiate in an aqueous solution of permanganate of potassium, $\frac{1}{4}$ per cent. or less.

8. Remove the brown of the permanganate of potassium in a 1 per cent. aqueous solution of oxalic acid.

9. Wash in water.

10. Alcohol, oil, chloroform balsam.

F. **Robertson's Modification of Heller's Myelin-sheath Stain.**—1. Harden in Weigert's fluorchrom-copper solution plus 4 per cent. of formaldehyde; in other words, use the mordant for neuroglia-fibers (page 370) eight to ten days.

2. Wash off in water.

3. Alcohol; imbed in celloidin.

4. Stain sections in a 1 per cent. solution of osmic acid half an hour in the dark.

5. Place in a 5 per cent. aqueous solution of pyrogallic acid for half an hour.

6. Differentiate in a $\frac{1}{4}$ per cent. aqueous solution of permanganate of potassium one to four minutes.

7. Remove brown color in 1 per cent. oxalic acid three to five minutes.

8. Alcohol, oil, balsam.

It is important to wash carefully in water between each of the staining steps.

G. **Myelin-sheath Stain for Frozen Sections** (Wright).—1. Fix in 4 per cent. formaldehyde solution.

2. Cut frozen sections.

3. Place in 50 per cent. alcohol for one minute, moving the section about in the fluid.

4. Place in 10 per cent. aqueous solution of ferric chloride for one minute.

5. Without washing transfer the section to a small quantity of a freshly prepared aqueous solution of hematoxylin for five minutes or longer. This is conveniently prepared by placing in a test-tube three or four small crystals of hematoxylin and 10 c.c. of distilled water, and heating over a flame until the solution is complete.

6. Wash quickly in water.

7. Differentiate by moving the section about in 10 per cent. aqueous solution of ferric chloride until the gray substance is well defined and the connective tissue of the pia mater appears yellow. Care should be taken not to differentiate too much and thus decolorize the myelin sheaths.

8. Wash *thoroughly* in a large quantity of distilled water or further decolorization will take place.

9. Dehydrate in alcohol.

10. Clear in origanum oil.

11. Press the section flat on the slide with blotting paper, or a pad of folded filter paper, and mount in xylol balsam.

Stains for Neuroglia-fibers.—It is possible to obtain a differential stain of the neuroglia-fibers in man by several different methods. The tissue must be as fresh as possible. The best results are obtained with tissues placed in the fixing solution within one hour after death. After four to six hours the results are only fair; after twenty-four hours they are practically *nil*. The peculiar property in the neuroglia-fibers on which the differential stain depends has undergone some chemical change or has disappeared. It is retained longest where the fibers are most numerous, as about the central canal.

Zenker's fluid, formaldehyde, or alcohol may be used as a fixative, according to the staining method preferred. Fixation in Zenker's fluid, followed by staining with phosphotungstic-acid hematoxylin, can be highly recommended and is much the quickest and simplest method. The Weigert method given here, and the only one he ever published, had been discarded by him in favor of a newer and better method, but, unfortunately, the secret of it perished with him.

For all the methods given it is imperative that the tissue should be cut into thin slices, not over 2 to 3 mm. thick, before it is placed in the fixing solution used.

No one of these methods stains neuroglia fibrils only. All stain fibrin, and all, with the possible exception of Weigert's, stain fibroglia and myoglia fibrils when these are freshly fixed.

A. **Mallory's Phosphotungstic-acid Hematoxylin Stain:**

1. Fixation in Zenker's fluid, 24 hours.
2. Running water, 24 "
3. Alcohol, 80 per cent., 24 "
4. Paraffin or celloidin imbedding.
5. Treat sections with iodin solution (Gram's iodin solution or a ½ per cent. alcoholic solution) to remove the mercury precipitate, five to ten minutes.
6. Alcohol, 95 per cent., several changes to remove iodin.
7. Water.
8. Permanganate of potassium, ¼ per cent. aqueous solution, for three to five minutes, sometimes ten to twenty minutes.
9. Wash in water.
10. Oxalic acid, 5 per cent. aqueous solution, five to ten minutes, sometimes longer.
11. Wash thoroughly in several changes of water.
12. Stain in phosphotungstic-acid hematoxylin for twelve to twenty-four hours.
13. Transfer directly to 95 per cent. alcohol, followed by absolute alcohol for paraffin sections, and dehydrate quickly.
14. Clear in xylol (filter-paper blotting method for celloidin sections) and mount in xylol balsam.

Neuroglia, fibroglia, and myoglia fibrils and fibrin blue, collagen fibrils reddish-brown; the coarse elastic fibrils sometimes stain of a purplish tint.

In step 13 the treatment with alcohol should not be prolonged over one to two minutes ordinarily, as the alcohol extracts the reddish color and destroys the sharp contrast between the different kinds of fibrils.

If after step 12 the sections are placed in a strong alcoholic solution (10 to 20 per cent.) of chlorid of iron for one to several minutes, followed by thorough washing in water, the collagen-fibrils and other reddish-stained structures are completely decolorized.

Xylol must be used as the clearing reagent, because after origanum and other oils the blue color fades.

Sections stained in phosphotungstic-acid hematoxylin keep for years if not unduly exposed to the light.

This same staining method can be used after formaldehyde fixation if the tissues are first carried through Zenker's fluid in the ordinary way, just as if the tissue were perfectly fresh.

B. **Weigert's Differential Stain for Neuroglia-fibers.**—*A.* Fix thin pieces of tissue, not over $\frac{1}{2}$ cm. thick, in a 4 per cent. solution of formaldehyde for at least four days.

B. Mordant in the following solution for four to five days in the incubator or for eight days at room-temperature:

Acetate of copper,	5 gr.;
Acetic acid, 36 per cent. solution,	5 c.c.;
Fluorchrom,	2.5 gr.;
Water,	ad 100 c.c.

Boil the fluorchrom and water in a covered dish, turn off the gas, add the acetic acid and then the acetate of copper; stir briskly until the latter is dissolved, then cool. The solution remains clear.

(Steps 1 and 2 may be combined by adding 4 per cent. of formaldehyde to the above solution; change on the second day; harden eight days.)

C. Wash off in water; dehydrate in alcohol; imbed in celloidin.

D. Reduction of copper salt in sections:

1. Place the sections, which must not be over .02 mm. thick, in a $\frac{1}{3}$ per cent. aqueous solution of permanganate of potassium for ten minutes.

2. Wash off with water.

3. Decolorize and reduce for two to four hours in the following solution, carefully filtered:

Chromogen,	5 gr.;
Formic acid (sp. gr. 1.20),	5 c.c.;
Water,	ad 100 "

to 90 c.c. of which are added just before using 10 c.c. of a 10 per cent. solution of sulphite of sodium.

The sections lose their color in a few minutes, but are best kept in the solution as long as above directed.

The sections can now be stained in the manner to be described, but the color of the fibers will be more intense if the following steps are added, and a slight yellowish contrast-stain is obtained for the ganglion and ependymal cells and for the larger nerve-fibers. This step has one disadvantage, however: the connective-tissue fibers stain blue after it.

E. Further reduction of copper salt:

1. Wash twice in water.

2. Place sections in a carefully filtered saturated (5 per cent.) aqueous solution of chromogen overnight.

3. Wash in water.

4. The sections are now ready for staining or may be preserved until wanted in—

80 per cent. alcohol,	90 c.c.
5 per cent. oxalic acid,	10 "

F. Staining of neuroglia-fibers:

1. Lift section from large dish of water on slide freshly cleaned with alcohol; blot with filter-paper (method recommended by Weigert for attaching sections to slide).

2. Stain in the following mixture:

Saturated solution of methyl-violet in 70–80 per cent. alcohol, (saturated with aid of heat; decanted when cold).	100 c.c.;
5 per cent. aqueous solution of oxalic acid,	5 "

The oxalic acid is added to render the preparations more lasting. The staining is practically instantaneous.

3. Wash off with normal salt solution.

4. Iodin solution: 5 per cent. iodid-of-potassium solution saturated with iodin. It is simply poured on and then off, as the reaction is instantaneous.

5. Wash off with water and blot with filter-paper.

6. Decolorize thoroughly in equal parts of xylol and aniline oil.

7. Wash repeatedly with xylol or the stain will not keep.

8. Canada balsam.

The sections keep better if exposed for from two to five days to diffuse light before being put away.

C. **Benda's Stain for Neuroglia Fibrils.**—*Hardening.*—1. Fix fresh material for at least two days in 90 to 93 per cent. alcohol.

2. Place thin sections (not over 5 mm. thick) in 10 per cent. nitric acid for twenty-four hours.

3. Two per cent. aqueous solution of bichromate of potassium, twenty-four hours.

4. One per cent. aqueous solution of chromic acid, forty-eight hours.

5. Wash in running water for twenty-four hours, harden in graded alcohols, imbed in paraffin.

Staining.—Iron-alizarin-toluidin-blue Stain.—1. Mordant the sections for twenty-four hours in a 4 per cent. solution of iron-alum.

2. Wash off in running water.

3. Twenty-four hours in dilute amber-yellow aqueous solution of sodium sulphalizarate.

4. Dip in water and blot with filter-paper.

5. Stain in a 1 per cent. aqueous solution of toluidin-blue, warm until steam rises, then let it stain about fifteen minutes in the cooling fluid; or stain one to twenty-four hours in cold toluidin-blue.

6. Dip in 10 per cent. acetic acid or in very dilute picric acid.

7. Dry with filter-paper and dip in absolute alcohol.

8. Differentiate in beech creasote about 10 minutes, controlling result with microscope.

9. Dry with filter-paper; xylol balsam.

Degenerations of the Nervous System.—The same methods apply to the study of degenerations in nervous tissue that apply elsewhere, except in the demonstration of fat. Both myelin and fat reduce osmic acid, so that the ordinary test for fat in the hardened tissues fails. Marchi and Algeri, however, have shown that after myelin has been

mordanted for eight days or over in Müller's fluid or other solution of the bichromates, it loses the property of reducing the osmic acid, while fat retains the property unimpaired. On this peculiarity is based their method for differentiating fat from myelin.

Marchi and Algeri's Method for Staining Fatty Degenerated Myelin-sheaths of Nerve-fibers.—1. Harden in Müller's fluid or in formaldehyde, followed by Müller's fluid, for eight days to three months.

2. Transfer tissue for five to eight days directly into the following solution:

Müller's fluid,	2 parts;
1 per cent. osmic-acid solution,	1 part.

3. Wash out thoroughly in water.
4. Dehydrate in alcohol.
5. Imbed in celloidin.
6. Clear in chloroform and mount in properly prepared chloroform balsam (see page 312).

EXAMINATION OF THE BLOOD.

The blood is conveniently obtained from the lobe of the ear by puncture with a Hagedorn needle. The part should be previously cleaned with alcohol and thoroughly dried. The free border of the lobe of the ear is preferable because it is convex, which is of advantage in making cover-glass preparations from small drops of blood.

Method of Counting the Red and White Blood-corpuscles.—The Thoma-Zeiss hemocytometer, or blood-counting apparatus, is generally employed, and consists of a glass slide, on which the blood-corpuscles are counted, and two graduated pipettes for mixing the blood and the diluting fluid. The counting slide has a square plate of glass cemented on its surface, and a circular opening in the center of this plate is nearly filled by a glass disc $\frac{1}{10}$ mm. thinner than the square plate which surrounds it. A series of horizontal and

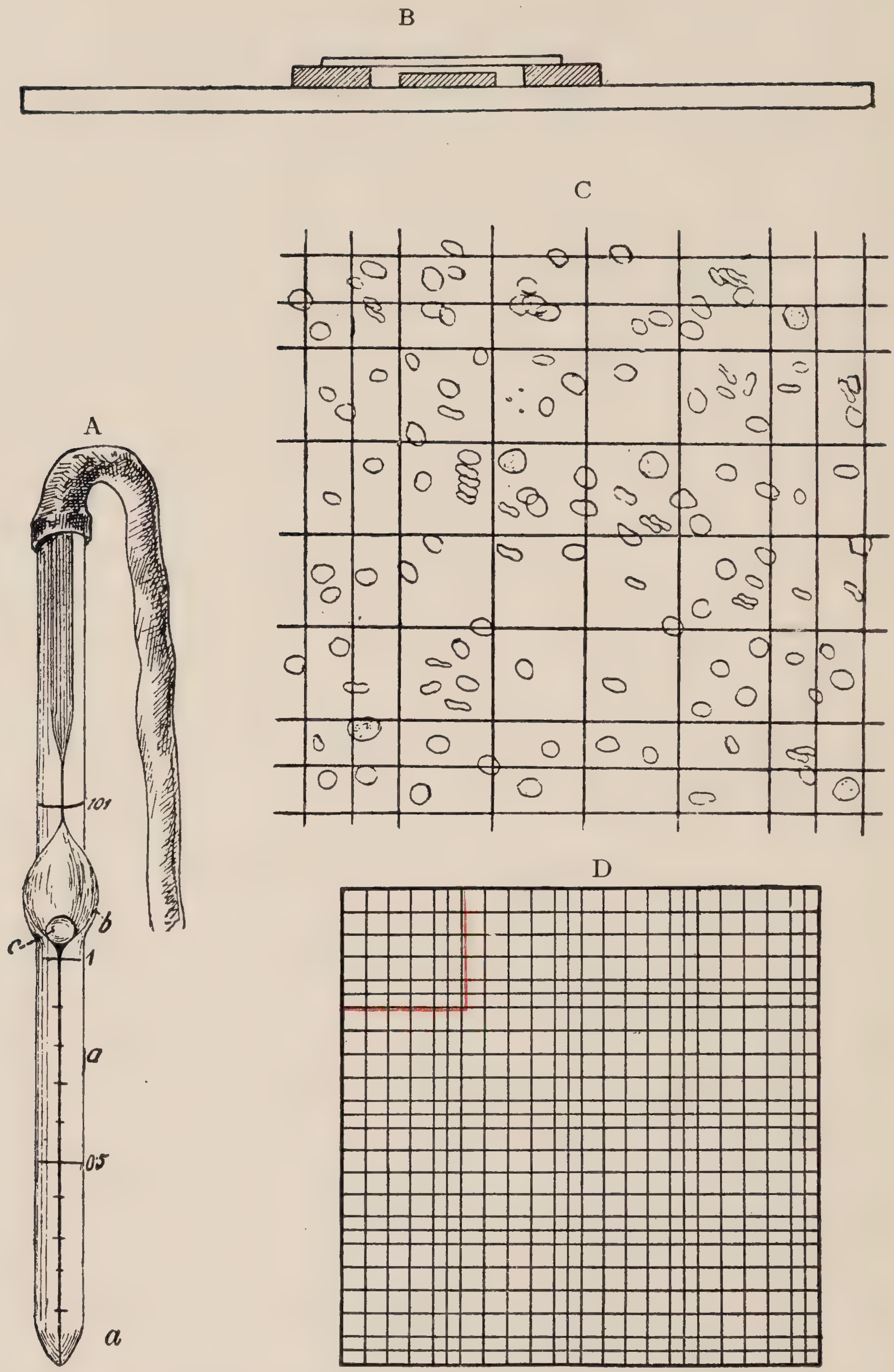


FIG. 128.—Thoma-Zeiss blood-counting apparatus (Limbeck): A, melangeur; *a*, capillary tube in which the blood is taken; *b*, chamber for mixing the blood with the diluting solution; *c*, glass ball to aid in mixing the blood with the diluting solution; B, cross-section of the chamber in which the blood is counted; C, section of the field on which the blood is counted, showing thirty-six squares; D, diagram of the whole field.

vertical lines on the surface of the disc divides it into squares, the sides of which are $\frac{1}{20}$ mm. long. Additional lines placed close together divide this surface into quadrants. Each quadrant contains sixteen of the small squares.

Each pipette consists of a capillary tube, which extends into an ovoid chamber above and is provided with a short piece of rubber tubing and a bone mouth-piece. The chamber contains a glass pearl which assists in mixing the blood and diluting fluid. For counting the *red blood-corpuscles* the blood is diluted 1 : 100 or 1 : 200 by means of the pipette which has the figures 101 over the line above the ovoid chamber. The diluting fluid recommended is that of Gowers, the formula of which is as follows:

Sodii sulphat.,	gr. 112;
Acid. acet.,	ʒv;
Aquæ,	℥iv.

With the mouth-piece of the rubber tube in the mouth, the blood is sucked up to the mark 0.5 or 1.0 on the capillary tube of the pipette, and then the tip of the tongue is placed firmly over the hole in the mouth-piece. This prevents the blood-column from sinking or air from entering below while the tip of the pipette is being wiped and immersed in the diluting fluid. This part of the test requires the utmost precision and avoidance of delay. It is necessary, therefore, to keep the eyes constantly fixed on the capillary tube in order to note any variation in the blood-column. Rapidly wipe the tip of the pipette to remove the blood from the outside, and then immerse the tip in the diluting fluid. Suck the fluid up to the mark 101, close the ends of the pipette with the thumb and middle finger, and shake the pipette for two minutes. If the ends of the pipette are not completely closed during this process, some of the fluid will escape. At the end of two minutes allow two drops to escape from the pipette before examination, because the fluid in the capillary tube is unmixed with blood. Then allow a drop to escape upon the central part of the counting slide. This drop should com-

pletely fill the depression after the cover-glass has been applied. A little practice is necessary in order to estimate the size of the drop required. A moderately thick cover-glass should be slid over or carefully laid upon the square raised surface, and pressure applied to the edges until the Newton color-zone can be seen between the cover-glass and the square raised surface beneath. Never press on the center of the cover-glass. Allow the blood-corpuscles to settle a minute or two before counting.

The corpuscles are estimated as follows: One side of a small square is $\frac{1}{20}$ mm. long; the enclosed square surface is $\frac{1}{400}$ mm. The distance between the cover-glass and the disc is .1 mm. which gives a cubic capacity of $\frac{1}{4000}$ c.mm. for each square. To estimate the number of corpuscles in 1 c.mm. of blood, multiply the number of corpuscles counted by 4000, and then by the number representing the amount of dilution, 100 or 200 as the case may be, and divide the result by the number of squares counted.

$$\frac{\text{Corp.} \times \text{dilution} \times 4000}{\text{Squares counted}} = \text{corpuscles in 1 c.mm.}$$

To avoid counting any of the corpuscles twice, always begin at the upper left-hand square of a quadrant and count four squares downward. Count all the corpuscles which touch the upper and left-hand lines of a square, together with the corpuscles in the square. Never count the corpuscles touching the right-hand or lower double lines of a quadrant. In order to make an accurate count it is necessary to count at least 1200 red corpuscles.

If air-bubbles are present when the cover-glass is applied, it is necessary to clean the slide and use a fresh drop of the diluted blood. Before beginning the count examine the various quadrants with a low-power objective, to see if the corpuscles are evenly distributed. If they are not, it will be because the blood is not thoroughly mixed, and the slide should be washed and the pipette well shaken. Before examining a second drop of the diluted blood shake the

pipette for two minutes as before. The results of three drops should be averaged.

For counting the *white blood-corpuscles* the pipette, graduated so as to give a dilution of 1 to 10 or 1 to 20, is employed. The white corpuscles are estimated in the same way as the red corpuscles, except that the dilution 10 or 20 is substituted for 100 or 200. This necessitates a fresh drop of blood. For a diluting fluid for counting the white corpuscles a $\frac{1}{3}$ or $\frac{1}{2}$ per cent. solution of acetic acid may be used. This solution destroys the red corpuscles.

After use the pipette should be cleaned with water followed by alcohol, and finally with ether until it is dry inside. Any coagulated albumen on the inside of the pipette may be removed by filling the pipette with the following solution and keeping it in the incubator for some hours:

5 gms. carbonate of sodium,
$\frac{1}{2}$ gm. pancreatin,
1000 c.c. water,
A few drops of chloroform.

In cleaning the counting slide water should be used. If it is necessary to employ alcohol for any reason, it must be used rapidly and the slide washed with water, because alcohol dissolves the cement by means of which the glass plates are attached to the slide.

Wright's Method of Counting the Blood-platelets.—The blood is mixed with a diluting fluid, in the proportion of 1 to 100, by means of the pipette used for counting red blood-corpuscles, and the counting is done in the ordinary blood-counting chamber with a high-power dry objective. In order to render the platelets more clearly visible, the specially thin cover-glass of Zeiss with central excavation is used (cover-glass No. 146, Zeiss catalogue). This may be obtained from Eimer & Amend, 205 Third Avenue, New York. The diluting fluid consists of three parts of an aqueous solution of potassium cyanide (1 : 1400) and two parts of an aqueous solution of brilliant cresyl-blue (1 : 300).

These two solutions must be kept in separate bottles, and mixed and filtered immediately before using. Of course the pipette should be well shaken before withdrawing the sample for counting. After the counting chamber is filled it is left at rest for ten or fifteen minutes, in order that the blood-platelets may all settle to the bottom of the chamber and be more easily and accurately counted.

The platelets appear as sharply outlined, round or oval or elongated, lilac-colored bodies, some of which form a part of a small sphere or globule of hyaline unstained substance. The red cells are decolorized and appear only as "shadows," so that they do not obscure the platelets. The nuclei of the white cells are stained a dark blue, the protoplasm light blue. If the technique is correct, there should be no precipitate in the preparation.

The cresyl-blue solution is permanent, but should be kept on ice in order to prevent the growth of yeasts. The cyanide solution should be made up at least every ten days. It is, of course, necessary that the solution be made from pure potassium cyanide which has not undergone decomposition. As already stated, the two solutions must be mixed and filtered *immediately* before using, because after filtration, if the mixture is allowed to remain exposed to the air for a short time, a precipitate will form in it. After the diluting fluid has been mixed with the blood in the pipette, however, no precipitate forms and, as the platelets do not quickly break up in the mixture, the counting may be done after some hours, if necessary. For example, a count immediately after filling the pipette was 258,000, and another count from the same filling of the pipette, made eighteen hours later, was 253,000.

A proper technique yields a remarkably even distribution of the platelets in the chamber. For all practical purposes the counting of the platelets in 100 small squares is sufficient, but for greater accuracy all 400 small squares should be counted, or 200 small squares in each of two fillings of the chamber.

The following is a sample count to show the even distribution of the platelets in the counting chamber:

Platelets in	Chamber No. 1.		Chamber No. 2.	
20 small squares	22	21	21	25
"	27	28	27	23
"	27	24	27	22
"	23	30	22	25
"	21	21	23	31
	120	124	120	126

By this method the platelet count of normal adults is found to vary from 226,000 to 367,000 per cubic millimeter, the general average being 297,000.

Cover-glass Preparations.—The blood must be spread extremely and uniformly thin. If this is done, the blood dries very quickly, and the red blood-corpuscles retain their shape and are not crowded together and lying over one another. To obtain such a result it is essential that the cover-glasses should be absolutely clean; that there should be no delay in bringing the cover-glass which has the drop of blood on its surface in contact with a second cover-glass; and that the drop of blood should be quite small. The following method gives the best results: The procedure is rendered much easier if some one is present to assist. This person places a finger beneath the lobe of the ear in order to raise it slightly without pressing upon it, and with a clean compress wipes away the blood as fast as it flows with a quick motion of the hand. This is done to prevent coagulation, which occurs very quickly, and prevents the drop of blood from spreading between the cover-glasses. It takes a little time for the blood to spread, the cover-glasses to be separated and laid down, and fresh ones picked up; and if, during this time, some one wipes away the blood as fast as it flows, much better preparations are obtained. If, in spite of this, as often happens, the blood coagulates about the opening, one end of the compress can be slightly moistened with water and passed over the opening and the surface dried quickly. The blood then flows freely again. A drop of

blood a little larger than a pin-head is sufficient. Grasp the edge of the cover-glass with a pair of spring forceps, pick up a second cover-glass with a pair of plain forceps. Both pairs of forceps must be especially prepared by having the inner surfaces of the points ground smooth. The cover-glass in the spring forceps is held horizontally just below the ear, and the other cover-glass, held with the other forceps, is touched lightly on the blood and immediately dropped on the first one. If the cover-glasses are dry and clean and the blood has not begun to coagulate, it spreads at once in a thin film between the glasses. The glasses are then drawn apart with a rapid sliding motion by means of the forceps, waved in the air a few seconds, and laid down with the blood-surface uppermost. The layer of blood cannot be too thin, but it can easily be too thick. The cover-glasses should never be pressed together to make the blood spread. Considerable practice is required before one becomes proficient. The specimens may be fixed by heat in a thermostat at a temperature between 110° and 120° C. (Ehrlich's method). This is objectionable on account of the time and apparatus required. A practical modification of this method is to heat the cover-glasses on a brass plate for an hour at a point on the plate where water boils. The plate should be about $\frac{1}{8}$ of an inch thick and from 15 to 18 inches long. It should be heated from one end to a constant temperature. Test the degree of heat with drops of water and select a part where the water boils. At a point nearer the flame it will be found that the water sputters and rolls about, indicating too high a temperature. After putting the cover-glasses, with the blood-side uppermost, upon the selected point, it is necessary to test the degree of heat from time to time, and perhaps to shift the cover-glasses.

Smear preparations on slides are easier to prepare than those on cover-glasses, and in many ways they are more satisfactory. All the precautions as to cleanliness described for cover-glasses are to be taken. The smear is made by placing a small drop of blood upon the slide near one end

and immediately spreading the blood by drawing the edge of the end of another slide through the drop and along the surface of the slide to its other extremity.

Methods of Staining.—Of the many staining fluids which have been employed to differentiate the white corpuscles, it is necessary to mention only two, which have been found to answer all purposes.

Ehrlich's Triple Stain.—The formula is as follows:

Orange G, saturated aqueous solution,	120 to 135 c.c.;
Distilled water,	100 "
. . . .	
Acid fuchsin, saturated aqueous solution,	65 "
Distilled water,	100 "
Absolute alcohol,	100 "
. . . .	
Methyl green, saturated aqueous solution,	125 c.c.;
Distilled water,	100 "
. . . .	
Absolute alcohol,	100 "
Glycerin,	100 "

The various ingredients are prepared separately as indicated by the dotted lines, and are afterward mixed gradually. The mixture must stand for several weeks before using. It is advisable to withdraw by means of a pipette some of the staining fluid from the middle portion without disturbing the bottom.

The cover-glass preparations should be stained from six to eight minutes, washed thoroughly with water, dried, and mounted in Canada balsam. The neutrophilic granules are stained violet; the eosinophilic, a bright red; the nuclei of the neutrophilic and the eosinophilic cells are a greenish-blue; the nuclei of the lymphocytes, a deep blue; the nuclei of the large mononuclear cells, a pale blue; the red

corpuscles, copper color; and the nuclei of the red corpuscles, if any be present, a more intense blue than the nuclei of the lymphocytes. For some unexplained reason this stain is not always uniform in its action.

It is sometimes difficult to distinguish a nucleated red corpuscle from a lymphocyte. It is well to remember, therefore, that the nuclei of red corpuscles stain more intensely than other nuclei, and have very sharply defined outlines, and by careful focusing it is seen that the surrounding stroma is stained the same color as the other red corpuscles.

Wright's Stain.—This staining fluid is an improvement on one devised by W. B. Leishman, because it requires only a few hours and an ordinary steam sterilizer for its preparation, while Leischman's required at least eleven days and the employment of a thermostat regulated at 65° C. Leishman deserves great credit for originating a method of staining blood-films and malarial parasites which combines the important "Romanowsky" staining with the great advantages of the methyl-alcohol method of Jenner. Wright's stain is applied in the same manner and gives the same results.

It is preferred to Ehrlich's stain, because it does not require the difficult and uncertain fixation of the blood-film by heat and because it gives constantly satisfactory results even in the hands of inexperienced workers.

This stain makes visible in the blood smear not only all that the Ehrlich's stain does, but more, for it gives the differential Romanowsky staining to mast-cells, blood-plates, certain degenerate products in the red corpuscles, and to malarial and other protozoan parasites, thus accomplishing at one and the same time all that which usually requires the employment of several special staining methods separately applied.

It is prepared as follows:

To a 0.5 per cent. aqueous solution of sodium bicarbonate add methylene-blue (B.X. or "medicinally pure") in the proportion of 1 gm. of the dye to each 100 c.c. of the solution. Heat the mixture in a steam sterilizer at 100° C. for

one full hour, counting the time after the sterilizer has become thoroughly heated. The mixture is to be contained in a flask, or flasks, of such size and shape that it forms a layer not more than 6 cm. deep. After heating, the mixture is allowed to cool, placing the flask in cold water if desired, and is then filtered to remove the precipitate which has formed in it. It should, when cold, have a deep purple red color when viewed in a thin layer by transmitted yellowish artificial light. It does not show this color while it is warm.

To each 100 c.c. of the filtered mixture add 500 c.c. of a 0.1 per cent. aqueous solution of "yellowish, water-soluble" eosin and mix thoroughly. Collect on a filter the abundant precipitate which immediately appears. When the precipitate is dry, dissolve it in methylic alcohol (Merck's "reagent") in the proportion of 0.1 gm. to 60 c.c. of the alcohol. In order to facilitate solution the precipitate is to be rubbed up with alcohol in a porcelain dish or mortar with a spatula or pestle.

This alcoholic solution of the precipitate is the staining fluid. It should be kept in a well-stoppered bottle because of the volatility of the alcohol. If it becomes too concentrated by evaporation and thus stains too deeply, or forms a precipitate on the blood smear, the addition of a suitable quantity of methylic alcohol will quickly correct such faults. It does not undergo any other spontaneous change than that of concentration by evaporation.

A most important fault met with in the working of some samples of this fluid is that it fails to stain the red blood-corpuscles a yellow or orange color, but stains them a blue color which cannot readily be removed by washing with water. This fault is due to a defect in the specimen of eosin employed. It can be eliminated by using a proper "yellowish, water-soluble" eosin.

Method of Staining Blood Films.—The films of blood which should be spread thinly are allowed to dry in the air.

1. Cover the film with a noted quantity of the staining fluid by means of a medicine-dropper.

2. After one minute add to the staining fluid the *same*

quantity of distilled water by means of the medicine-dropper, and allow it to remain for two or three minutes, according to the intensity of the staining desired. A longer period of staining may produce a precipitate. Eosinophilic granules are best brought out by a shorter period of staining. The quantity of diluted fluid on the preparation should not be so large that some of it runs off.

3. Wash the preparation in water for thirty seconds, or until the thinner portions of the preparation become yellow or pink in color.

4. Dry and mount in balsam.

Films more than a few hours old do not stain as well as fresh ones.

Microscopical Appearances in Blood-films Stained with Wright's Stain.—The *red cells* are orange or pink in color. Polychromatophilia and punctate basophilia or granular degeneration are well brought out. The nucleated red cells have deep-blue nuclei, and the cytoplasm is usually of a bluish tint.

The *lymphocytes* have dark purplish-blue nuclei and robin's-egg-blue cytoplasm in which a few dark-blue or purplish granules are sometimes present.

The *polynuclear neutrophilic leucocytes* have a dark-blue or dark lilac-colored nucleus, and the granules are usually of a reddish-lilac color.

The *eosinophilic leucocytes* have blue or dark lilac-colored nuclei. The granules have the color of eosin, while the cytoplasm in which they are imbedded has a blue color.

The *large mononuclear leucocytes* appear in at least two forms. Each form has a blue or dark lilac-colored nucleus. The cytoplasm of one form is pale blue and of the other form is blue with dark-lilac or deep-purple-colored granules, which are usually not so numerous as are the granules in the polynuclear neutrophilic leucocytes.

The *mast-cells* appear as cells of about the size of polynuclear leucocytes with purplish or dark-blue stained, irregular-shaped nuclei, and with cytoplasm, sometimes bluish, in which numerous coarse spherical granules of

variable size are embedded. These granules are of a dark purple color and may appear almost black.

The *myelocytes* have dark-blue or dark lilac-colored nuclei and blue cytoplasm in which numerous dark-lilac or reddish-lilac-colored granules are imbedded. In leukemia more color differences are brought out among the leucocytes than by the ordinary methods of staining.

The *blood-platelets* are well stained. In the best preparations they generally appear as round or oval blue bodies with smooth or finely irregular margins, containing, chiefly in their central portions, many small violet to purplish granules. They are usually of a diameter of one-third to one-half that of the red blood-corpuscles. They frequently occur in groups and masses. Occasionally elongated forms are seen which may have a length of six or more times their width. These are, according to Wright's observations, the detached larger pseudopods of the giant cells of the bone-marrow, just as the smaller platelets are detached smaller pseudopods or fragments of the larger pseudopods of the same cells.

Method of Examining Blood without Drying or Fixation.—A small quantity of a 1 per cent. aqueous solution of brilliant cresyl-blue is dried upon a slide in such a manner that a thin, transparent film of the dried dye is obtained. Upon this a small drop of blood is placed and immediately covered with a cover-glass. The blood is spread out in as thin a layer as possible under the cover-glass, using gentle pressure if necessary. The plasma immediately dissolves the dye on the surface of the slide, and in the course of some minutes the following appearances will be seen upon examination with an oil immersion objective: the white blood-corpuscles are stained blue and show all details of their structure; the blood-platelets remain intact and appear as irregular-shaped hyaline bodies, each with a round, blue-staining, somewhat granular central structure. In the interior of a few of the red blood-corpuscles a blue-staining reticulum of delicate, contorted filaments will be seen. In anemic blood many more corpuscles contain this reticulum. The blood-corpuscles do not become abnormal in shape, but retain their natural cup shape. Fibrin does not form.

The preparation may be kept under observation for hours before marked disintegrative changes occur in the formed elements. Instead of the dye mentioned on page 385, Wright's blood-stain may be used.

Schultze's Oxydase Reaction.—Many cells possess an oxydizing ferment, which they disclose by forming synthetically naphthol blue when they are treated first with α-naphthol and then with dimethyl-p-phenylendiamin. The method is particularly useful for differentating myelocytes from cells of the lymphocyte series; the myelocytes give a positive reaction, while the lymphocytes are negative.

The reaction is carried out as follows (two solutions are required):

1. One gram of α-naphthol is heated to boiling in 100 c.c. of distilled water until it melts and floats in the water. Pure potassium hydrate (about 1 c.c.) is added until all the naphthol is dissolved. The solution appears at first yellow, later yellowish brown.

2. One per cent. aqueous solution of dimethyl-p-phenylendiamin (Merck), made at room temperature and filtered.

Cover-glass preparations fixed in formaldehyde vapor or frozen sections of formaldehyde fixed tissue are placed in solution (1) for a few minutes, and then in solution (2) for a similar length of time; they should be moved gently back and forth in the solutions.

The preparations after being washed in distilled water should be mounted and examined in water or glycerin jelly. The granules, which exhibit the oxydase reaction, are stained deep blue. The preparations are not permament.

METHODS OF FIXING AND EXAMINING SPECIAL ORGANS AND TISSUES.

Tissues which are to be hardened should be obtained as fresh as possible. For this reason autopsies rarely furnish such perfect material as is obtainable from experimental lesions in animals or from surgical operations. Still, most of the pathological material comes from autopsies, and it is

encouraging to know that very good work can often be done with tissues not fixed until twenty-four hours or even more after death. The most valuable autopsies are those which are freshest, and in which but one etiological factor has been concerned, so that the relation between the cause and the lesion produced is uncomplicated and can be readily grasped and understood.

The choice of the proper fixing reagent varies with the tissue, the lesion, and the use to which the material is to be put. It is advised as a routine procedure to preserve tissues in two fluids: in Zenker's fluid for general histological study, including both the injurious agents of all sorts and the inflammatory reactions to them; in formaldehyde for the preservation of fat and myelin, and certain substances to which it may be desirable to apply chemical tests. With these two fixatives properly applied it is possible to go a long way in pathological histology. Orth's fluid may be substituted for Zenker's fluid, but is distinctly not so good. Alcohol is required for the preservation of certain substances, such as glycogen and urate of sodium crystals, and corrosive sublimate fixation is necessary for certain special stains for mucus.

It is imperative that pieces of tissue for histological study should be placed in the proper fixative as soon after the removal of the organs from the body as possible, so that the surface will not dry or the blood and other fluids escape from the vessels. Do not wash off the surface with water. The tissues should almost invariably be cut into thin slices, not over 2 to 4 mm. thick.

In preserving tissues it is very important to use enough of the fixing reagent—ten to fifteen times as much as there is tissue. It is advised to harden tissue in flat-bottomed glass dishes and to stir them occasionally, so that they may come in contact with fresh fluid.

After Zenker fixation the best stain to use for general histological study is methylene-blue and eosin. For class use alum hematoxylin and eosin make a fairly satisfactory substitute, but do not demonstrate any bacteria present. The other useful stains are phosphotungstic acid hematoxylin,

the aniline blue method for collagen fibrils, and Verhoeff's elastic tissue stain. After formaldehyde fixation the most interesting results are obtained by staining frozen sections with Scharlach R and alum hematoxylin.

These methods of fixation and staining are applicable to most of the tissues listed below, and constitute the routine stains for almost all organs outside of the central nervous system, and even there they are often useful.

Acute Inflammatory Exudations; Granulation-tissue.—The elements in acute inflammatory exudations which require preservation are chiefly leucocytes of different sorts, serum, fibrin, and red blood-corpuscles. The best general fixative for them all is Zenker's fluid. It not only preserves perfectly the characteristic nuclei of the leucocytes, but also the cytoplasm, which stands out sharply in contrast-staining with eosin. The albumin of the serum is coagulated into a finely granular material. The fibrin and red blood-corpuscles stain brilliantly with eosin.

Lung.—In the preservation of the lungs it is important to save portions that have not been squeezed, so that the relations of the exudations may not have been changed or the alveoli compressed. Thin slices are usually preferable to cubical pieces, and should be cut with a very sharp knife, so as not to compress the tissue, and dropped immediately into the fixing fluid, before the contents of the bronchi and of small cavities have had time to run out. An emphysematous lung is so delicate that it is usually better to inject a whole lung through the bronchi with the fixing fluid or to snip out small pieces with scissors. Zenker's fluid and formaldehyde are the most useful fixatives.

Bone-marrow and Spleen.—On account of the similarity in the cellular content of these organs they are considered together. They may be studied by both smear and section methods.

Sections.—The pieces of tissue to be fixed should be about 2 mm. thick. The sections are to be cut in paraffin and are to be as thin as possible. For general purposes, fixation in Zenker's fluid and staining by the eosin-methy-

lene-blue method are recommended. Other recommendations are:

1. For the study of erythroblasts and the formation of red blood-corpuscles: fixation in corrosive sublimate and staining by eosin soluble in alcohol and alkaline methylene-blue.

2. For the demonstration of the granules of myelocytes and leucocytes: fixation in corrosive sublimate and staining by Wright's blood-staining fluid undiluted, then washing in water, dehydrating in acetone, clearing in oil of turpentine, and mounting in turpentine colophonium. The Biondi-Heidenhain triple stain may also be used.

Schridde's Method of Staining the Granulations of Myelocytes and Leucocytes in Sections.—The tissue is best fixed in Orth's fixing fluid, but other fixatives may be used. The sections should not be thicker than 5 μ and should be fixed to the slide with Mayer's albumen mixture. They are stained for twenty minutes in Giemsa's stain, diluted with distilled water in the proportion of two drops of the stain to each cubic centimeter of water. The mixture must be freshly made before using. When the staining is completed the preparation is washed in water, the excess of water removed with filter-paper, and the section immediately placed in pure acetone. If the acetone extracts color from the preparation, it is impure and should not be used. The section is then cleared with toluol or xylol and embedded in neutral xylol balsam. Care should be taken not to allow the sections to become dry from the rapid evaporation of the acetone.

The neutrophile granulations are stained a violet-red; the eosin granulations red; the granulations of the mast-cells dark blue, and the granulations in the cytoplasm of the megakaryocytes violet-red. All nuclei are blue and the red blood-corpuscles grass-green. The connective tissue is of a pale red color.

Wright's Method for the Differential Staining of the Blood-platelets and the Giant Cells (Megakaryocytes) of the Bone-marrow.—The tissue should be absolutely fresh.

It is fixed in a 4 per cent. aqueous solution of formaldehyde or in a saturated solution of corrosive sublimate in 0.9 per cent. salt solution. Tissue that has been decalcified is not suitable. The sections are cut in paraffin and should not be more than 7 microns thick. They are stained while affixed to the slide by Mayer's egg-albumen method.

The staining fluid and the mode of its preparation are described below.

The staining, clearing, and mounting are carried out as follows:

1. Equal parts of the staining fluid and distilled water are mixed in a small wineglass and immediately poured on to the slide. The measuring is conveniently done by means of a small pipette provided with a rubber bulb. At least 2 c.c. of the freshly diluted staining fluid are thus spread out over the slide, which should be supported upon some object in such a way as to prevent the fluid from running off. The spreading out of the fluid in a layer is important, because it facilitates the evaporation of the alcohol, whereby the staining elements slowly precipitate out of solution and, while doing so, stain the tissue elements. This precipitate appears as a yellowish, metallic scum which slowly forms on the surface of the mixture. The diluted staining fluid is allowed to act for about fifteen minutes, when the preparation is immediately washed in water. The exact time required for the best results has to be determined for each batch of the staining fluid. The proper staining of the preparation may be judged by examining it under a low magnifying power by artificial light after pouring back the diluted staining fluid into the wineglass. The staining is stopped by washing the preparation in water, when the cytoplasm of the giant-cells has acquired a bright red color and the fibrils of the reticulum begin to take on a red color also. If the staining is found not sufficiently intense, the diluted staining fluid is poured back on the preparation and allowed to act longer. Over-staining and the formation of a black-red granular precipitate on the preparation occur if the diluted staining fluid is allowed to act longer than a certain time.

2. Dehydrate in pure acetone.

On account of the great volatility of acetone, some care is necessary to prevent the drying of the preparation, which should be avoided.

3. Clear in xylol or pure oil of turpentine.

4. Mount in a thick solution of colophonium in xylol or pure oil of turpentine.

Before mounting the preparation the superfluous turpentine should be removed, because this reagent rapidly takes up water from the air, and thus may cause the clouding of the preparation or the fading of the stain.

The solution of colophonium is made by saturating a quantity of turpentine with powdered colophonium, and keeping the filtered solution in the paraffin embedding oven until it has evaporated to the required consistence.

The use of acetone instead of alcohol for dehydrating is an important feature of the method, for the latter spoils the characteristic staining of the granules in the giant-cells and platelets.

The *staining fluid* is composed of 1 part of a modified methylene-blue solution and 10 parts of an 0.1 per cent. solution of water-soluble eosin in pure methyl alcohol.

The solution of methylene-blue is prepared as follows: One gram of methylene-blue "med. pur." is dissolved as thoroughly as possible in 100 c.c. of an 0.5 per cent. aqueous solution of sodium bicarbonate in an Erlenmeyer flask. The flask and its contents are then placed in an ordinary steam sterilizer and kept at 100° C. for one hour and a half, counting the time after the steaming has become vigorous. When cool, the mixture is filtered and the filtrate is the modified blue solution. It must be of a well-marked purple color when viewed in a thin layer by the yellow transmitted light of an ordinary incandescent electric bulb. This color appears only after cooling.

Variations in the solutions of the blue and of the eosin may require that the proportions above given be changed slightly. An excess of eosin delays the appearance of the scum on the surface of the diluted staining fluid, and pro-

longs the time required for staining. On the other hand, an excess of the modified blue component hastens the appearance of the scum, and may cause overstaining and the granular precipitate to form on the preparation.

The blood-platelets typically appear as rounded bodies, more or less jagged in outline, and composed of a hyaline, blue-staining substance, in which are embedded, chiefly in the central portions, fine red to purplish granules. The cytoplasm of the giant-cells shows the same structure and staining peculiarities. The sections should be examined by an incandescent electric light in order to bring out the colors to the best advantage. By this method all grades of transition can be shown between pseudopod-like processes of the giant-cells or detached masses of giant-cell cytoplasm and blood-platelets.

Smear preparations may be made either upon slides or cover-glasses, and stained by Wright's blood-stain, as in the case of blood-smears. For the best results the preparation should not be allowed to dry, but should be stained immediately while still wet, and, after staining, dehydrated with absolute alcohol, cleared with xylol, and mounted in balsam in the same manner as a section affixed to the slide. A longer period of staining than that directed for blood-smears is usually desirable.

Special Methods for Smear Preparations from Bone-marrow.—Thoroughly tease a small bit of the marrow in a few drops of blood-serum and from this mixture prepare the smear preparation, as in the case of a blood-smear. The preparation, however, must not be allowed to dry, but is fixed immediately by methyl-alcohol (one minute) *while still wet.* It is then covered for three to five minutes with a mixture of equal parts of Wright's blood-stain and distilled water. This mixture must have been prepared immediately before use. After staining, the preparation is not allowed to dry, but is washed in water, dehydrated with acetone, cleared with oil of turpentine, and mounted in turpentine colophonium. Instead of methyl-alcohol, corrosive sublimate, Zenker's fluid, or a 1 per cent. solution of osmic acid may be

used for fixation, each being allowed to act about one minute, when the preparation is to be washed in water, covered with 95 per cent. alcohol for one minute, rinsed in water, and then treated as indicated. It is important that the preparation be not allowed to dry at any stage of the process.

By these methods many of the finer details of the marrow-cells are brought out much better than by the usual smear method.

Kidney.—Zenker's fluid and formaldehyde as fixatives answer most purposes, but alcohol is required to preserve glycogen and certain crystalline deposits. The Scharlach R. stain, after formaldehyde fixation, has to a large extent replaced Flemming's solution. Fixation by boiling is still used to demonstrate an albuminous exudate in the capsular space. The general staining methods already recommended will be found the most satisfactory.

In cases of chronic nephritis the capsule should not be peeled from those parts kept for microscopical purposes.

Paraffin embedding is generally to be preferred for the kidney, especially when lesions of the glomeruli are present.

Gastro-intestinal Tract.—Portions of the stomach or intestine should be hardened as soon after death as possible for satisfactory study, because the gastro-intestinal tract so rapidly undergoes post-mortem changes. It has been recommended in appropriate cases, where an autopsy is allowable, to inject the stomach with the desired fixing solution by means of a rubber tube as soon after death as is permissible. Under no circumstances should the surface of the intestine or stomach be washed with water. Use either normal salt solution or some of the fixing solution. It is important to keep the tissue flat while hardening. This can usually be done by laying it with the peritoneal surface down on thick filter-paper, to which it readily sticks. Sometimes it is necessary to pin the specimens down at the edges on flat pieces of cork. Do not let the surface dry before the specimen is placed in the fixing solution. Zenker's fluid can be highly recommended as a fixative, but alcohol is sometimes to be preferred.

Liver.—Fat is most easily and satisfactorily demonstrated by the Scharlach R. stain after formaldehyde fixation. Necrosis of liver-cells is best shown by the eosin-methylene-blue stain after fixation in Zenker's fluid. The necrotic cells stand out of a deep pink color in sharp contrast to the other cells. The aniline blue stain is especially useful in the study of the lesions associated with chronic passive congestion and with amyloid deposit.

For obtaining the iron reaction with hemosiderin in cases of pernicious anemia, and for the reactions of amyloid, harden in formaldehyde or alcohol.

For general histological study Zenker's fluid will be found exceedingly useful.

The bile-capillaries may be demonstrated by the same method that is used for neuroglia fibrils—namely, fixation in Zenker's fluid, following by staining in phosphotungstic-acid hematoxylin. The treatment with permanganate of potassium and oxalic acid must be more prolonged than usual, however, otherwise the albuminous granules in the cytoplasm will stain too deeply and obscure the capillaries.

For Eppinger's elaborate method, see *Ziegler's Beiträge*, vol. xxxi.

Pancreas.—Much interesting and valuable work has been done recently on the histology of the pancreas, especially with reference to the cytoplasmic granules in the different kinds of cells of the ducts, glands, and islets. Some attempt has been made to apply the methods to the lesions of the pancreas, more particularly to those associated with the syndrome known as diabetes mellitus. The results so far obtained are promising and encourage further study along the same lines.

For routine microscopic study of the pancreas, fixation in Zenker's fluid and staining by the eosin-methylene-blue method are recommended. The zymogen granules do not stain intensely, as in the glands of the stomach and intestine after this procedure, but require special methods to render them prominent. Staining with phosphotungstic acid hematoxylin âfter fixation in formaldehyde is sometimes useful.

Fixation in formaldehyde is also useful for certain other purposes, such as the examination for fat, hemosiderin, and amyloid.

As the best method for staining all the various granules in the cells of the pancreas, Bensley recommends that used for mitochondria (see page 333).

For the specific granules of the A and B cells in the islets, the best technique in Bensley's opinion is the neutral gentian stain after fixation in chrome sublimate.[1]

A. Fix in the following solution (Lane's):

Potassium bichromate,	2.5	grams.
Mercuric chlorid,	5.0	"
Distilled water,	100.0	"

It is Zenker's fluid minus the acetic acid, as the latter dissolves both mitochondria and the characteristic granules of the islet cells.

The stain used is Bensley's neutral gentian, the name given to the neutral dye obtained when a solution of gentian violet is precipitated by its equivalent of a solution of Orange-G. The dye is prepared as follows:

A. Gentian violet, 1 gram in 25 c.c. of water.

B. Orange G., 1 gram in 25 c.c. of water.

Add A to B, shaking gently, until practically complete precipitation has taken place. Filter and wash with water at once. Drain and dry. Dissolve residue in 25 c.c. of absolute alcohol. For staining, add the stock solution of the neutral compound to 20 per cent. alcohol until a solution having the color of a good hemalum solution is obtained. Allow this solution to stand twenty-four hours to permit the excess of dye to separate out, when it may be employed for staining as follows:

1. Stain in neutral gentian violet solution twenty-four hours.
2. Blot between several layers of filter paper.
3. Dehydrate in acetone.

[1] For other methods of fixing and staining the islet cells consult these papers: Bensley, R. R., "Studies on the Pancreas of the Guinea Pig," *Amer. Jour. of Anat.*, 297–388, xii., 1911; Lane, M. A., "The Cytological Characters of the Areas of Langerhans," *Amer. Jour. of Anat.*, vii., 1907.

4. Place sections in toluol.
5. Differentiate in

Absolute alcohol,	1 part;
Oil of cloves,	3 parts;

6. Wash with toluol and mount in balsam.

In the stain for mitochondria the granules in the A cells are stained deeply red, those in the B cells green. After the neutral gentian violet stain, followed by staining in acid fuchsin, the granules of the A cells are stained red, those of the B cells, violet.

Bone and Cartilage.—Excellent work can be done after hardening in alcohol, and fixation in it is generally recommended for all infectious processes in bone. The histological structure is, however, better preserved in Zenker's or Orth's fluid. In decalcifying bone, after proper fixation, thin pieces should be taken, not more than 2 to 4 mm. thick, so that the process may be finished as quickly as possible. While tubercle bacilli will stain readily after being twenty-four or even forty-eight hours in 5 per cent. nitric acid, it is impossible to stain them after they have been subjected to the same strength of nitric acid for four days. (For details in regard to decalcification see page 277.)

Celloidin is preferable to paraffin for imbedding. Besides a simple stain with alum-hematoxylin, double stains of the latter with neutral carmine or eosin are sometimes advantageous. The best pictures with carmine as the contrast-stain are obtained by staining first in alum-hematoxylin, washing twelve to twenty-four hours, and then staining in the neutral carmine. The carmine stains decalcified bone and osteoid tissue red. Phosphotungstic-acid hematoxylin will sometimes be found useful, especially when cartilage is present, because it stains the intercellular substance, both of bone and of cartilage, pink, while the nuclei are stained blue. The ground substance of cartilage, especially in new-growths, often stains so intensely with alum-hematoxylin that the nuclei are quite obscured. For the same reason chlorid of iron hematoxylin is often useful because it does not stain the ground substance.

The following method is recommended for differentiating cartilage from bone:

Schaffer's Safranin Method.—Decalcify with nitric acid.

1. Stain sections a half to one hour in an aqueous solution of safranin, 1 : 2000.

2. Wash in water.

3. Place for two to three hours in a $\frac{1}{10}$ per cent. solution of corrosive sublimate.

4. Examine in glycerin, or, if permanent specimens are desired, pass very quickly through alcohol, blot with filter-paper, further dehydrate, and clear for a long time in bergamot or clove oil, and mount in xylol balsam. This is a double stain: cartilage, orange; bone, uncolored; connective tissue and marrow, red.

None of the methods above given has proved reliable in the study of rickets and of osteomalacia for differentiating osteoid from true bone-tissue. In important cases, therefore, it is advisable to use an old knife, and to cut sections of the undecalcified tissue after imbedding thoroughly in celloidin.

Schmorl's methods of demonstrating the lacunæ and canaliculæ of bone in sections can be highly recommended.

Method A.—1. Fix, preferably in Müller's fluid, formaldehyde, or Orth's fluid; do not use corrosive sublimate solution.

2. Decalcify by the slower methods—namely, Ebner's or Thoma's, or in Müller's fluids 100 c.c. plus nitric acid 3 c.c.

3. Imbed in celloidin; paraffin is objectionable.

4. Place the sections for at least ten minutes in water to get rid of the alcohol.

5. Stain for five to ten minutes or longer in saturated solution of thionin in 50 per cent. alcohol, 2 c.c., water, 10 c.c., or in Nicolle's carbolthionin solution.

6. Wash in water.

7. Place in a saturated aqueous solution of picric acid for one-half to one minute.

8. Wash in water.

9. Place in 70 per cent. alcohol for about five to ten minutes until no more dense clouds of color are given off.

10. Dehydrate in 95 per cent. alcohol.
11. Clear in oleum origani cretici.
12. Xylol balsam.

Bone substance yellow to yellowish-brown; bone lacunæ and canaliculæ dark brown to black; cells red. Fat-cells after fixation in Müller's fluid reddish violet. Osseous tissue stains a deeper yellow than osteoid tissue. Canaliculæ stain in osseous tissue, but not in osteoid tissue unless the thionin solution is made alkaline by the addition of 1 or 2 drops of ammonia. (This solution cannot be recommended for general use.)

This method is not a true stain, but resembles Golgi's method; a precipitation of coloring-matter takes place in the lacunæ and canaliculæ; it also takes place to a considerable extent in other narrow spaces in the tissues, and often is very disturbing. It can be gotten rid of to some extent without injury to the stain by leaving the sections in step 8 in the water for half an hour. The canaliculæ are now usually brownish red to red, and the bone substance blue to colorless. In this case it is often best to stain the sections first in alum hematoxylin to bring out the nuclei.

Method B gives good results with the bones of children only. 1. Harden in Müller's fluid or in Orth's fluid, followed by Müller's for six to eight weeks, or for three to four weeks in the thermostat; take very thin pieces of tissue.

2. Wash off in water, and decalcify in Ebner's solution.

3. Wash thoroughly in running water.

4. Harden in alcohol; imbed in celloidin; cut sections very thin.

5. Stain in Nicolle's carbolthionin, or better in the alkaline (NH_4OH) thionin solution given above, for three minutes.

6. Transfer to a saturated aqueous solution of phosphotungstic or phosphomolybdic acid (use glass or platinum needle) for a few seconds or longer. The sections become blue, green, or gray in color.

7. Water five to ten minutes until they acquire a sky-blue color.

8. Place in dilute ammonia (1–10) for three to five minutes to fix the color.

9. Transfer directly to 90 per cent. alcohol; change several times to get rid of the ammonia.

10. 96 per cent. alcohol.

11. Clear in carbol xylol.

12. Xylol balsam.

If the ground-substance is stained too deeply by the alkaline thionin solution, treat the sections with acid alcohol for five minutes, followed by water before dehydrating. The borders of the lacunæ and canaliculæ stain bluish black; the ground-substance of bone clear to greenish blue; cellular elements a diffuse blue color. In rachitic bones the canaliculæ are brought out only in osseous tissue.

Skin.—Much of the material for the study of lesions of the skin is obtained during life by means of the knife or scissors. Fixation in absolute alcohol is often advisable, especially when it is desired to stain bacteria, mastzellen, plasma-cells, and elastic fibers. The staining methods for these tissue elements will be found on pages 333–345. For Unna's innumerable stains for degenerated connective-tissue fibers, elastic fibers, etc., the reader is referred to his numerous articles on technique in the *Monatsheft f. prakt. Dermatologie.*

For many skin-lesions, especially those in which blood-vessels play a more or less prominent part, Zenker's fluid is advisable. Clear tissues in oil of cedar wood rather than in chloroform for paraffin imbedding as it does not render them quite so brittle as chloroform does.

In the examination of hairs or scales of epidermis for bacteria and fungi it is important first to remove the fat from them by means of equal parts of alcohol and ether. They are then examined in 40 per cent. caustic potash, which, by clearing up the cells, brings out the organisms and spores quite distinctly. Heating the potash over a small flame hastens the process, but is a somewhat risky proceeding; soaking in the solution over night is better. Examine the preparation with most of the light excluded.

Preparations may be made in certain cases by touching the cover-slip to the surface of the lesion, drying, and passing through the flame. After removing the fat by means of alcohol and ether, stain as with ordinary cover-slip preparations.

Unna's method is to rub up the scales of epidermis in a little glacial acetic acid between two slides, which are then drawn apart and quickly dried over the flame. After removing the fat by means of alcohol and ether the slide preparations are stained in borax-methylene-blue.

For staining the various vegetable parasites of the skin Malcolm Morris recommends the following method, which he claims is the best one yet devised, as it avoids the use of the hydrate of potash:

1. Ether or alcohol and ether equal parts.
2. Stain in a solution of 5 per cent. gentian-violet in 70 per cent. alcohol, five to thirty minutes.
3. Iodin solution, one minute.
4. Aniline, or aniline plus 2 to 4 drops of nitric acid.
5. Aniline.
6. Xylol.
7. Xylol balsam.

The most suitable medium for the growth of the various ringworms is the following, due to Sabouraud:

Agar-agar,	1.30;
Peptones,	.50;
Maltose	3.80;
Water,	100.

Instead of test-tubes, Erlenmeyer flasks are used, so as to get a large flat surface for the growth to spread over from the point of inoculation in the center. The most favorable temperature for growth is 30° C.

Museum Preparations.—Specimens intended to be preserved for the museum should generally be gotten into pretty good shape by trimming and dissecting before they are placed in the hardening reagent. Of the liver or other large organs and tumors, sections several centimeters thick are generally preferable to the whole specimen. The usual custom in the past has been to wash the specimen for a number of hours or over night in running water, to get rid of the blood, and then to preserve in 80 per cent. alcohol.

This method preserves form and relations well, but is nearly valueless for preserving colors.

Since the introduction of formaldehyde, from which at first much was expected in the way of faithful fixation of the normal colors of gross preparations, numerous attempts have been made to improve on the results obtainable with formaldehyde alone. Of the methods advocated, the following from Virchow's laboratory seems the most promising, and can be highly recommended:

Kaiserling's Method of Preserving the Natural Colors in Museum Preparations.—1. Fixation for one to five days in—

Formaldehyde,	200 c.c.;
Water,	1000 "
Nitrate of potassium,	15 grams;
Acetate of potassium,	30 "

Change the position of the specimen frequently, using rubber gloves to protect the hands from the injurious effect of the formaldehyde. The time of fixation varies with the tissue or organ and size of the specimen.

2. Drain and place in 80 per cent. alcohol one to six hours, and then in 95 per cent. alcohol for one to two hours, to restore the color, which is somewhat affected in the fixing solution.

3. Preserve in—

Acetate of potassium,	200 grams;
Glycerin,	400 c.c.
Water,	2000 "

Exposure to light gradually affects the colors. The process of fixation should be performed in the dark, and the specimens when preserved should be kept in the dark except when on exhibition.

If it seems desirable to cut a thin slice from the face of a specimen, this should not be done until the preparation has been in the preservative fluid two weeks. The specimen

may then be placed in alcohol for one to two hours to brighten up the colors.

It is advisable to add camphor, thymol, carbolic acid (one per cent.), or some other preservative to the third solution to prevent the growth of molds.

Pick has modified the steps of Kaiserling's method as follows:

1. Fixation up to five days in—

Water,	1000 c.c.;
Formaldehyde,	50 c.c.;
Carlsbad salt,	50 gm.

2. Eighty to 85 per cent. alcohol.

3.	Water,	900 c.c.;
	Glycerine,	540 c.c.;
	Acetate of sodium,	270 gm.

For display purposes the preparations preserved by these methods are often mounted permanently in gelatin. A jelly is made by adding ten parts by weight of gelatin to the third solution in which the specimens are ordinarily kept. The same procedure should be followed as in the making of Kaiser's glycerine jelly for the mounting of histological sections. For a preservative add one per cent. of carbolic acid. Formaldehyde is often used for this purpose, but in spite of it, or owing to its evaporation, ferments present in the tissues (as shown by L. J. Rhea) often result in liquefaction of the jelly in the course of weeks to months.

Owing to the discoloration which sometimes takes place in the jelly and the difficulty of remounting the tissues, L. W. Williams advises using only a layer of the jelly sufficient to attach the specimen to the back of the jar, and filling up the space in front with the third solution, which can easily be renewed. By this method the specimen is held firmly in place and is viewed through a clear, colorless medium.

PATHOLOGICAL PRODUCTS.

Cloudy Swelling; Albuminous Degeneration.—The increase in the relative number of the albuminous granules of the cytoplasm of the various tissue-cells in pathological processes is usually determined by examination of the fresh material, either macroscopically from the appearances on section, or microscopically from teased preparations or frozen sections mounted in salt solution. The organ as a whole (and therefore the individual cells) usually shows some increase in size. The nucleus is generally more or less obscured if the process is at all marked. According to Israel, the cloudiness must be recognizable with low powers and in places where the cells are massed together. The diagnosis should not be based on the appearances of single cells.

The chemical properties of the albuminous granules are the following: they disappear on treatment with dilute acetic acid (1–2 per cent. solution usually); they are not dissolved by chemical substances which dissolve fat (absolute alcohol, ether, chloroform, etc.); and they do not stain with osmic acid. The acetic-acid test is the one usually employed.

Albuminous degeneration can also be studied in sections of tissues hardened in certain of the fixatives and stained with diffuse colors. For this purpose hardening in Zenker's fluid and staining in eosin and methylene-blue or in alum-hematoxylin and eosin can be highly recommended.

Fat.—This term includes a variety of fat-like substances, of which the three most common and important in the animal kingdom are olein, palmitin, and stearin, the glycerinates of oleic, palmitic, and stearic acids. Under certain conditions the free fatty acids themselves and their lime-salts may be present. Among the fat-like substances belongs also the myelin of the nerve-sheaths. The pigments of the ganglion-cells and of many other cells contain fat, and hence react like fat, but the fat and the pigment can be separated.

These various forms of fat differ somewhat in their reaction to certain reagents used as tests. They are all insoluble in dilute acids and alkalies, but are soluble in strong alcohol,

in ether, and in chloroform. Osmium tetraoxid is reduced by olein and oleic acid, by myelin, and the fat in certain pigments, but is not reduced by the palmitic and stearic components of body-fat unless the tissue after exposure to the osmium tetraoxid is placed in dilute alcohol; if placed in strong alcohol, the reduction takes place imperfectly or not at all. Myelin differs from the other fat substances in that it can be fixed by a chrome salt so that it will no longer reduce the osmium tetraoxide.

Scharlach R. is the strongest of a group of fat stains of which Sudan III. has, perhaps, been the most used until recently. These stains have the common property of dissolving readily in all fats, including myelin and the lipochrome of ganglion-cells. The staining, which is purely physical in nature, depends simply on the fact that fat is a better solvent of the stain than the alcohol is, and takes it up from it.

The common tests for fat are the three following: As all these fatty substances are unaffected (at least not for some months) by formaldehyde, it is becoming more and more customary to apply the tests, especially the Scharlach R. stain, to frozen sections after fixation in that reagent.

1. **Acetic Acid.**—The test may be applied to teased preparations or to frozen sections. The acetic acid is generally employed in a 1 or 2 per cent. solution, of which a few drops are placed at the edge of the cover-slip and drawn under by means of a bit of filter-paper placed at the opposite edge. Albuminous granules disappear optically while fat-droplets persist.

2. **The Osmium Stain for Fat.**—A 1 per cent. solution may be applied like the acetic-acid test to teased preparations or to frozen sections. It is more usual, however, to fix very thin sections (not over 1 mm. thick) in a 1 per cent. solution of osmium tetraoxid for one to three days, or, better still, in a combined solution, such as Flemming's or Marchi's, containing it.

Flemming's solution should be allowed to act from two to four days if the tissue is from 2–3 mm. thick, and then the pieces of tissue should be thoroughly washed in running

water for twenty-four hours before being placed for several days in dilute and then in strong alcohol.

Marchi's method was intended for differentiating fat from myelin (see page 277), but the solution employed by him may be used for staining fat in ordinary tissues. Place small pieces of tissue in it for five to eight days, wash thoroughly in running water, and place in dilute (50 to 70 per cent.) alcohol for several days before transferring to strong alcohol.

Marchi's method, carried through in the manner just described, succeeds perfectly with tissues fixed in formaldehyde.

Osmium reduced by fat is soluble in ether, turpentine, xylol, and toluol, but is not dissolved by alcohol, chloroform, or oil of cloves. Steensland adds oleum origani cretici to this list and recommends it for clearing celloidin sections. Imbedding in celloidin is not contra-indicated, as the alcohol probably protects the osmium from the injurious action of the ether. For the paraffin method clear in chloroform, and mount in properly prepared chloroform balsam (see page 312).

3. **The Scharlach R. Stain for Fat.**—The solution of Scharlach R. most commonly used has been a saturated one in 70 per cent. alcohol. It requires staining overnight in order to obtain the best results. Recently, G. Herxheimer has advised a saturated solution in—

Seventy per cent. alcohol,	50 c.c.;
Pure acetone,	50 "

Staining takes place very quickly and is intense and sharp. The solution can be highly recommended and should take the place of any other yet proposed.

Cover-slip Preparations.—1. Fix in the vapor of formaldehyde for five to ten minutes.

2. Stain in the alcohol-acetone solution of Scharlach R. for two to five minutes.

3. Dip for an instant in 70 per cent. alcohol.

4. Wash in water.

5. Counterstain with alum hematoxylin or methylene-blue.

6. Wash in water.

7. Mount in glycerin or glycerin jelly.

Sections.—1. Make frozen sections of formaldehyde-fixed tissue.

2. Dip for an instant in 70 per cent. alcohol.

3. Stain in the alcohol-acetone solution of Scharlach R. for two to five minutes.

4. Wash quickly in 70 per cent. alcohol.

5. Counterstain in alum hematoxylin.

6. Wash thoroughly in water.

7. Mount in glycerin or glycerin jelly.

The staining should always be done in a tightly stoppered bottle, because with any evaporation of the alcohol a precipitation of the staining material immediately takes place.

If, after staining with alum-hematoxylin, the sections are put into a 1 per cent. aqueous solution of acetic acid for three to five minutes, the color of the nuclei is a clearer blue, in better contrast with the red color of the fat, and the staining is sharper.

Benda's Stain for Fat Acid Crystals.—In a solution of copper acetate the crystals of the free fatty acids and of the fatty acid salts of lime-stain blue. Benda recommends fixation in Weigert's copper-fluorchrom-acetic-acid-mordant plus 10 parts of formaldehyde for two to four days at 37° C. Frozen sections are then to be cut, counterstained in Scharlach R. and alum-hematoxylin, and mounted in glycerin.

This method has been modified by Fisher, and later by Klotz, as follows:

1. Fix the tissue and precipitate the fatty-acid radical in the following solution for one to twenty-four hours:

Chromalum,	2.5	grams.
Formaldehyde, 4 per cent.,	100	c.c.

Dissolve by boiling; while cooling add—

Glacial acetic acid,	5	c.c.
And then powdered neutral acetate of copper,	5	grams.

2. Wash thoroughly in water.

3. Cut sections on freezing microtome.

4. Stain sections in a saturated solution of hematoxylin in 60 per cent. alcohol for six hours.

5. Wash in water and treat with the following fluid (Weigert's decolorizing fluid) until the tissue becomes a light brown, while the sites of the fatty-acid radical remain black:

Potassium ferricyanide,	2.5 grams.
Borax,	2.0 "
Water, distilled,	100.0 c.c.

6. Water, alcohol, balsam.

After washing in water the sections may be stained with Scharlach R. and mounted in glycerin.

Cholesterin crystals are recognized by their shape. On the addition of concentrated sulphuric acid the crystals turn yellow and then rose-color. Treated with a little iodin, followed by concentrated sulphuric acid, they become colored violet, changing gradually to blue, green, and red.

Necrosis.—Necrosis in tissues is generally recognized by two features: either by the disappearance of the nuclei, although the cell-outlines may be visible, so that the nuclear stain is no longer possible, or by the presence of irregular, larger or smaller masses, generally supposed to be due to a fragmentation or breaking-up of the chromatin, which stain intensely with nuclear stains. The disappearance of the nucleus is not synchronous with the death of the cell, but begins some twenty-four hours later, so that it is really evidence of changes following necrosis. It follows from the above that the microscopic evidence of necrosis is best studied in sections of tissues hardened in fixatives which favor nuclear staining, such as Zenker's fluid, formaldehyde, etc. Teased preparations and frozen sections of fresh tissue are much less useful.

For the study of sections from hardened material double stains with alum-hematoxylin and eosin, or, still better, with eosin followed by Unna's alkaline methylene-blue solution, after Zenker's fixation, are very useful, for the reason that the necrotic areas usually stain rather deeply with the diffuse

stain, and are thereby brought out sharply. This is particularly true of necroses of the liver.

For rendering the fragmented nuclei prominent the same methods may be followed as for mitosis. A fuchsin stain washed out by picric acid in the alcohol will often give excellent results.

Caseation is probably a tissue-change following local necrosis. Macroscopically and microscopically it resembles harder or softer cheese. Under the microscope it appears as coarsely or finely granular masses which have more or less completely lost the original tissue-structure. The chemical changes which have taken place have not been studied. Fibrin may or may not be present. Caseous tissue possesses no peculiar staining reactions. Fragmented nuclei are frequently present in it, especially in the peripheries of the areas.

Fibrin.—Fibrin usually appears as delicate, transparent, slightly refractive threads which are often closely matted together so as to form large masses. More rarely it appears as coherent masses of the finest granules, as homogeneous glassy lumps, or as thin sheets. The characteristic reaction for fresh fibrin is that it quickly swells up and optically dissolves in very dilute acetic acid.

Fibrin is well brought out in sections of hardened tissues by a double stain of alum-hematoxylin and eosin, or of eosin followed by Unna's alkaline methylene-blue solution, especially if the specimens have been fixed in Zenker's fluid. Two other stains which bring it out with great sharpness are phosphotungstic-acid-hematoxylin and the aniline-blue method for collagen fibrils.

Weigert's Differential Stain for Fibrin.—1. Harden in alcohol.

2. Stain sections in lithium carmine (see page 326).
3. Stain in Weigert's aniline-methyl-violet three seconds.
4. Wash off with normal salt solution.
5. Weigert's iodin solution a few seconds.
6. Wash off with water.
7. Decolorize in aniline oil and xylol equal parts.

8. Wash off with three changes of xylol.
9. Xylol balsam.

To stain sections fixed in formaldehyde or in a chrome salt, place them in a $\frac{1}{3}$ per cent. aqueous solution of permanganate of potassium for ten minutes, wash in water, and reduce in a 5 per cent. aqueous solution of oxalic acid for two to three hours or longer. Then wash thoroughly in water.

The fibrin and those bacteria which are stained by Gram are stained blue. The nuclei are red if the decolorization is carried far enough. It can easily be watched under the low power of the microscope. The method is not always successful, especially with tissues which are old. Besides the fibrin, certain forms of hyalin are often stained by this method.

Differential stains for fibrin are also obtained by the chlorid of iron hematoxylin stain (page 324) and by the connective-tissue stain (page 338). The former is applicable after any fixing reagent, the latter only after Zenker's fluid.

The phosphotungstic-acid-hematoxylin method is also useful if all the steps are followed out as for neuroglia fibrils.

Mucin.—The term "mucin" is applied to a proteid substance having certain chemical reactions, and also to certain other substances which give the same reactions, but do not belong to the proteids. These various substances of secretory and degenerative origin cannot be distinguished microscopically, and have been investigated but little chemically. The reactions in common are the following: they dissolve in water to form a slimy fluid; they are precipitated from slightly alkaline solutions by acetic acid; the fresh precipitate dissolves in alkalies and in neutral salt solutions. Acetic acid, usually employed for this purpose in a 1 or 2 per cent. solution, precipitates mucin in the form of threads or granules. This reaction with fresh tissues has long been the main test for mucin. The acetic acid is drawn under the cover-slip by means of filter-paper placed at the opposite edge. The preparation should be mounted in water, not in

salt solution, which may hinder or entirely prevent the reaction from taking place. Of late certain color reactions have become prominent. Mucin is coagulated into threads by alcohol or corrosive sublimate, and in this form can be stained by a number of staining reagents. Alum-hematoxylin under certain conditions will stain mucin. According to P. Mayer, these conditions depend on a certain degree of ripeness of the solution, on the presence of enough alum to keep the nuclei from staining deeply, and, most important of all, on the absence of any free acid. This is difficult to manage, unless the solution is carefully neutralized, on account of the acid properties of alum. Mayer, therefore, recommends staining the sections in muchematein.

Mayer's Muchematein.—

Hematein,	0.2 grams;
Chlorid of aluminum,	0.1 "
Glycerin,	40 c.c.;
Water,	60 "

Rub up the hematein with a few drops of glycerin and the chlorid of aluminum, and dissolve the mixture in the glycerin and water. Mucin appears blue: the other tissue elements are not stained.

Various aniline dyes have been recommended for staining mucin: those most favorably spoken of are methylene-blue (Orth), Bismarck brown (P. Mayer), thionin (Hoyer), polychrome methylene-blue (Unna), and toluidin-blue. The drawback to most of the aniline stains is that they are quickly extracted by the alcohol used for dehydrating. On this account P. Mayer highly recommends Bismarck brown, because permanent mounts can be easily made with it. It is not extracted by alcohol, and it does not fade in Canada balsam like many of the others.

Hardening in corrosive sublimate and imbedding in paraffin are generally recommended as preferable to hardening in alcohol and imbedding in celloidin. Stain sections for five to fifteen minutes in a rather dilute aqueous solution of the dye chosen. Of Bismarck brown use a saturated aque-

ous solution, and stain, if necessary, twenty-four hours. With thionin, toluidin-blue, and polychrome methylene-blue metachromatic stains are obtained; the mucin is colored red, the rest of the tissue blue. Two special methods for staining mucin are given in detail:

Hoyer's Thionin Stain.—Mucin, red; everything else, blue. 1. Harden in corrosive sublimate, followed by alcohol.

2. Paraffin sections are passed through xylol, chloroform, and 95 per cent. alcohol to free them from paraffin, and are then placed in a 5 per cent. aqueous solution of corrosive sublimate for three to five minutes.

3. Stain in a dilute solution of thionin for ten to fifteen minutes.

4. Alcohol.

5. Clear in the mixture of the oils of cloves and thyme.

6. Turpentine oil or oil of cedar.

7. Balsam.

Before the staining the sections must not be treated with iodin solution to get rid of the precipitate of mercury, because it spoils the staining.

Unna's Polychrome Methylene-blue Stain.—1. Fix in alcohol. Stain paraffin or celloidin sections in polychrome methylene-blue five to ten minutes or longer.

2. Wash in acidulated water.

3. Fix in 10 per cent. solution of bichromate of potassium half a minute.

4. Wash in water.

5. Dry on slide with filter-paper.

6. Decolorize in aniline plus 1 per cent. hydrochloric acid (a few seconds only).

7. Wash off with oil of bergamot.

8. Balsam.

Nuclei blue, mucin, cartilage, and amyloid red.

Pseudo-mucin dissolves in water to form a slimy material, and is precipitated from its solutions by alcohol in thread-like masses which are again soluble in water. It is not affected by acetic acid. Pseudo-mucin is found in certain ovarian and other tumors.

Colloid and Hyalin.—The terms colloid and hyalin are not yet sharply limited to definite chemical substances. The term colloid was originally applied to the homogeneous substance found in the thyroid gland, but has been broadened to include various substances of a similar appearance. The term hyalin is still more indefinite, but its use may be said to be applied most generally to those homogeneous substances which stain deeply with various stains, in contradistinction to those which, like colloid, show no marked affinity for staining reagents after ordinary fixatives.

Unquestionably, numerous substances of different chemical composition and of varying origin have been grouped under these two titles because of their physical and optical characteristics—namely, that they occur as glassy, refractive, homogeneous, occasionally colored gelatinous or firm masses. Chemically, very little that is definite is known about them, and they possess no peculiar chemical reactions. Several attempts have been made to classify them in accordance with their reactions to various staining reagents.

Von Recklinghausen applied the term colloid to all the homogeneous, transparent-looking substances, including mucin, amyloid, etc., and reserved the term hyalin for a special group, which, according to him, is characterized by the following peculiarities: it resembles amyloid in physical characteristics, but does not react to iodin; it stains deeply with acid dyes, such as eosin and acid fuchsin.

Ernst has recently endeavored to differentiate two groups of hyaline substances, colloid and hyalin, by means of their reaction to Van Gieson's picro-acid fuchsin solution. According to him, true hyalin stains with acid fuchsin alone, and appears of a deep-red color, while colloid, of which the typical example is found in the thyroid gland, stains with both picric acid and acid fuchsin, so that it appears of an orange or yellowish-brown color. He has also tried to prove that all colloid is derived from epithelial cells, while all hyalin comes from connective tissue or from blood-vessels.

According to Von Kahlden, these differential staining reactions with Van Gieson's mixture claimed by Ernst for col-

loid and hyalin are by no means justifiable, because true colloid often stains a deep red. Furthermore, Unna has shown that in the skin connective-tissue cells can give rise to the so-called true hyalin, of which part is acidophilic and part basophilic, while the intercellular substance gives rise to colloid.

The last attempt to classify the various homogeneous substances on the basis of their reactions to dyes, apparently the only method possible at present, has been made by Pianese as a result of his studies of the various degenerative processes occurring in cancer-cells. He used a special fixative (see p. 276) and five different staining methods (see p. 304, methods III. A. and B, IV., V., and VI.). Of these methods, III. B. is the best, because it gives a characteristic color to each substance—hyalin, brick-red; colloid, bright green; mucin, clear sky-blue; and a substance resembling amyloid, a dark reddish-violet. Besides these distinct reactions for colloid, hyalin, mucin, and a substance resembling amyloid, he found others less definite; one of these he calls pseudo-mucin and another pseudo-colloid. As a basis for his studies he took the reactions of amyloid, mucin (intestine), colloid (thyroid gland), and hyalin (hyalin remains of ovarian follicles, hyaline degeneration of renal glomeruli), with the same stains after fixation in his own hardening mixture.

The above brief historical statement is considered necessary to show the present views in regard to these various, more or less indefinite, homogeneous, transparent substances. For demonstrating them after the usual hardening reagents, of which alcohol and corrosive sublimate are perhaps the best, a double stain with alum-hematoxylin and eosin is very useful. Certain of the homogeneous substances stain deeply with eosin; others, like the transparent drops and masses occasionally found in the walls of the blood-vessels of the brain, stain with hematoxylin. Sometimes good results can be obtained with Weigert's fibrin stain or with carbol-fuchsin. The most generally useful stain, aside from alum-hematoxylin and eosin, is probably Van Gieson's mixture.

The hyalin in liver-cells in alcoholic cirrhosis stains deeply by the eosin-methylene-blue method after fixation in Zenker's fluid. The color varies from blue to red, depending, to some extent at least, on the freshness of the tissue. It stains deep blue in phosphotungstic-acid-hematoxylin preparations.

1. Stain deeply in alum-hematoxylin.
2. Wash in water.
3. Stain three to five minutes in a saturated aqueous solution of picric acid, to which is added enough of a saturated aqueous solution of acid fuchsin to give it a deep-red color. The effect of various proportions is sometimes useful.
4. Wash in water.
5. Alcohol.
6. Oleum origani cretici.
7. Balsam.

The transparent homogeneous substances usually stain from orange to deep red in color; connective tissue, red.

Unna's Method for Hyaline and Colloid Material.—*A.* Harden in alcohol. 1. Acid fuchsin (2 per cent. aqueous solution) five minutes.

2. Saturated aqueous solution of picric acid two minutes.
3. Saturated alcoholic solution of picric acid two minutes.
4. Wash off in alcohol.
5. Oil, balsam.

Hyaline and connective-tissue fibers, red; colloid of thyroid gland, yellow; protoplasm, yellow.

B. To show acidophilic and basophilic hyaline: 1. Water-blue (2 per cent. aqueous solution), twenty to thirty seconds.

2. Water.
3. Carbol-fuchsin one to two minutes.
4. Water.
5. Alcohol slightly tinged with iodin.
6. Pure alcohol.
7. Oil, balsam.

Nuclei, keratin, and large hyaline masses, cherry red; connective-tissue fibrillæ, protoplasm, and small hyaline bodies, blue.

For finer work the methods of Pianese should be used.

Keratohyalin (*Unna*).—1. Stain sections in a fairly old alum-hematoxylin solution until they are over-stained.

2. Place in a very weak solution of permanganate of potassium (about 1 : 2000) for ten seconds.

3. Dehydrate and decolorize in alcohol.

4. Oil, balsam.

An isolated stain of the granules of keratohyalin is obtained, blue-black in color.

In like manner a 33 per cent. solution of sulphate of iron acting for ten minutes, or a 10 per cent. solution of chlorid of iron for a few seconds, will produce the same effect. Ordinarily, sections are stained deeply in alum-hematoxylin, and decolorized with acetic acid and alcohol or with hydrochloric acid and alcohol.

Glycogen.—Glycogen is a carbohydrate of slightly varying composition, occurring in cells, more rarely in the intercellular tissue, either diffusely or more commonly in the form of larger and smaller masses and granules of a transparent homogeneous appearance. It is demonstrated microchemically by means of its reaction with iodin, which stains it brown. It is easily differentiated from amyloid by the fact that with the exception of the glycogen from certain sources, such as cartilage-cells, it is readily soluble in water and does not give the iodin-sulphuric-acid reaction.

In consequence of its property of dissolving readily in water the aqueous Lugol's solution of iodin cannot be employed for staining glycogen in fresh tissues. Instead, a thick solution of gum arabic containing 1 per cent. of Lugol's solution must be used, or, better still, equal parts of glycerin and Lugol's solution, in which the sections are more perfectly cleared.

For sections hardened in absolute alcohol the same methods may be used, but better results, and practically permanent mounts, can be obtained by the method of Langhans. Lugol's solution is used for staining the sections, because after hardening in alcohol the glycogen is much less soluble in water than in the fresh state. The iodin-glycerin mixture would probably be better.

1. **Langhans' Iodin Stain.**

1. Stain paraffin sections in Lugol's solution.

2. Dehydrate in 1 part of tincture of iodin to 3 or 4 parts of absolute alcohol.

3. Clear in oleum origani cretici.

The sections are to be preserved in oil. Even a ring of balsam around the cover-slip will cause the color to fade. Other oils are not so good.

2. **Lubarsch's Iodin-Hematoxylin Stain.**

1. Fixation in absolute alcohol.

2. Stain paraffin sections for five minutes in the following solution, which should be filtered and carefully protected from sunlight:

Delafield's hematoxylin,	2;
Lugol's solution,	2;
Water,	1.

3. Absolute alcohol, xylol, xylol balsam.

4. Expose to daylight one to two days. Glycogen brown, nuclei blue.

Lubarsch's Gentian-violet Stain.—Alcohol fixation; paraffin imbedding.

1. Stain with Meyer's alcoholic carmine solution, differentiate in acid alcohol, wash off in absolute alcohol.

2. Stain in aniline oil gentian-violet for one to two minutes, warming slightly if necessary.

3. Wash quickly in water.

4. Gram's solution of iodin on section continuously for five to ten seconds.

5. Dry thoroughly with filter-paper.

6. Dehydrate and differentiate in aniline-oil xylol (2 to 1) or in pure aniline oil.

7. Wash thoroughly in xylol and mount in balsam.

Nuclei red; glycogen dark blue to violet. It is advisable to expose the sections to daylight for one to two days. The preparations will keep up to one year.

Best's Carmine Stain.—Fix tissues in alcohol: formaldehyde and corrosive sublimate are not so good.

Imbed in celloidin, which prevents the glycogen from dissolving in water. Paraffin and frozen sections should not be used.

The stock carmine solution is made as follows:

Carmine,	2.0 grams.
Potassium carbonate,	1.0 "
Potassium chlorid (KCl),	5.0 "
Aq. dest.,	60.0 c.c.

Boil gently and cautiously for several minutes.

After cooling add—

Liq. ammon. caustic.,	20.0 c.c.

In tightly stoppered bottles this solution will keep and be available for staining glycogen for two months in winter, and for about three weeks in summer.

The staining method is as follows:

1. Stain sections deeply with alum hematoxylin.
2. Decolorize with acid alcohol, if necessary.
3. Wash thoroughly in running water.
4. Stain sections for five minutes in the following solution:

The above carmine solution (freshly filtered),	2.0 c.c.
Liq. ammon. caustic.,	3.0 "
Methyl alcohol,	3.0 "

5. Differentiate in—

Alcohol abs.,	80.0 c.c.
Methyl-alcohol,	40.0 "
Aq. dest.,	100.0 "

from three to five minutes, changing the fluid occasionally until it remains uncolored.

6. Wash off in 80 per cent. alcohol.
7. Alcohol, oil, balsam.

Glycogen red, nuclei blue. The method also stains the peptic cells of the stomach, the corpora amylacea of the nervous system, and sometimes the mucin in goblet-cells and the granules of mast-cells.

Caution: Do not put the sections into water after steps 4 or 5, because the carmine will diffuse out of the specimens.

Amyloid Infiltration.—Amyloid is a combination of an albuminous body with chondroitin sulphuric acid. It is insoluble in water, alcohol, ether, and dilute acids, and is not digested by pepsin and hydrochloric acid. It is distinguished from the other homogeneous substances, except glycogen, by the fact that it is stained mahogany-brown by iodin in solution. The reaction is particularly useful as a test on fresh gross material. If a section containing amyloid be quickly and lightly stained in Gram's iodin solution and then transferred to sulphuric acid, the color of the amyloid will usually change at once or in a few minutes from red, through violet, to blue. Sometimes the color turns simply of a deeper brown. Several of the aniline dyes give almost as perfect characteristic color reactions for amyloid as iodin, and are perhaps better for the purposes of histological study. Any of these differential stains may be used with fresh or hardened tissues. Alcohol as a hardening reagent gives the best results, but the other fixatives may be employed. Unfortunately, good permanent mounts cannot be made with any of the characteristic stains, so that the ordinary double stains of alum-hematoxylin with eosin or Van Gieson's mixture will often be found of the greatest help in studying the distribution of amyloid. The aniline-blue connective-tisue stain can also be highly recommended because it stains amyloid light blue, so that it stands out in marked contrast to the red of the liver-cells.

Iodin Reaction for Amyloid.—1. Stain sections in a weak solution of iodin (Lugol's solution diluted until of a clear yellow color) for three minutes.

2. Wash in water.

3. Mount and examine in water or glycerin.

If the tissue reacts strongly alkaline, a condition which may result from post-mortem decomposition, the color reaction with iodin will not take place. In such cases the tissue or the sections of it should be treated with dilute acetic acid before applying the test. The normal reaction of amyloid

with iodin may be increased by treating the section after staining with dilute acetic acid.

Langhans' Method for Obtaining Permanent Mounts with Iodin.—1. Harden in alcohol and stain in Mayer's alcoholic carmine solution.

2. Stain sections in Lugol's solution five to ten minutes.

3. Dehydrate quickly in 1 part of tincture of iodin to 3 or 4 parts of absolute alcohol.

4. Clear and mount in oleum origani cretici.

The color is said to keep remarkably well. Other oils or balsam cause it to fade quickly. The staining in Lugol's solution may be omitted, as the tincture of iodin usually stains the amyloid sufficiently deeply.

Iodin and Sulphuric-acid Reaction.—1. Stain quickly and lightly in dilute Lugol's solution.

2. Treat with sulphuric acid, either concentrated or dilute (1 to 5 per cent.), on the slide or in the staining dish. Strong hydrochloric acid may be used in the same way.

The change of colors from red to blue already spoken of usually occurs within a few minutes, but occasionally does not take place at all.

The following substances give reactions with the above iodin tests:

1. *Cholesterin crystals* are stained rather dark with dilute iodin solution, and turn a beautiful blue color at the edges on the addition of strong sulphuric acid.

2. The *corpora amylacea* in the prostate and central nervous system stain brown with the dilute iodin solution.

3. *Starch-granules* stain blue with dilute iodin solution.

4. *Cellulose* stains yellow with iodin. If washed and treated with strong sulphuric acid, it turns blue where the acid touches it.

For the reactions with the aniline dyes the sections must be free from celloidin.

Reaction with Methyl-violet.—1. Stain frozen sections of fresh or of formaldehyde or alcohol fixed tissue in 1 per cent. methyl-violet three to five minutes.

2. Wash in a 1 per cent. aqueous solution of acetic acid.

3. Wash thoroughly in water to remove all trace of acid.

4. Examine in water or in glycerin.

The stain will keep for some time if mounted in a saturated solution of acetate of potash or in levulose. Other methods are to stain in aniline-methyl-violet and to wash out in a 1 per cent. solution of hydrochloric acid, or to stain in a strong solution of methyl-violet to which acetic acid is added, and to wash out in water. The amyloid is stained violet-red, the tissue blue. Sections of tissues imbedded in celloidin will not give the reaction unless the celloidin is removed. The color reaction shows best when the light for the microscope is taken from a white source.

Reaction with Iodin-green.—1. Stain fresh or hardened sections in a $\frac{1}{3}$ per cent. aqueous solution of iodin-green for twenty-four hours.

2. Wash in water.

3. Mount in water or glycerin.

Amyloid, a violet-red; tissue, green. Stilling claims that the reaction is surer than with methyl-violet.

Reaction with Bismarck Brown and Methyl-violet (Birch-Hirschfeld).—1. Stain in a 2 per cent. alcoholic solution of Bismarck brown for five minutes.

2. Wash in absolute alcohol.

3. Wash in distilled water ten minutes.

4. Stain in a 2 per cent. aqueous solution of methyl-violet five to ten minutes.

5. Wash in dilute acetic-acid solution.

6. Wash thoroughly in tap water.

7. Mount in levulose.

Amyloid, red; tissue, brown.

Mayer's Stain for Amyloid.

1. Transfer paraffin sections without previous treatment directly from the knife to a warmed (40° C.) half per cent. aqueous solution of gentian-violet for five to ten minutes.

2. Wash in water and differentiate in a 1 per cent. solution of acetic acid for ten to fifteen minutes.

3. Wash thoroughly in water.

4. Transfer to a half concentrated aqueous solution of alum. Wash off in water.

5. Transfer sections to slide and allow the water to evaporate.

6. Remove paraffin and clear with xylol. Mount in xylol balsam.

Pigmentation.—The various pigments found in the human body under normal and pathological conditions may be divided into three groups:

1. **Hematogenous pigments**, derived from the coloring matter of the blood.

(*a*) *Hemoglobin* and *methemoglobin:* soluble in water and alcohol, not absolute; occur as yellowish to yellowish-brown granules and droplets; stain deeply with eosin after proper fixation; occur in hemoglobinuria, etc.

(*b*) *Parhemoglobin:* a form of hemoglobin; crystallizes like it, but is insoluble in alcohol.

(*c*) *Hematoidin=bilirubin:* contains no iron; is insoluble in water, alcohol, and ether; dissolves in chloroform; occurs as yellow or brown amorphous material or as crystalline rhombic plates and needles. Is found in extravasations of blood.

(*d*) *Hemosiderin:* occurs as bright-colored, yellowish-brown and brown granules and masses; gives iron reaction; is insoluble in water, alcohol, and ether; is found in extravasations of blood, in the liver in pernicious anemia, etc.

(*e*) *Melanin:* occurs as dark-brown or black granules and masses; does not give iron reaction; is found in malaria in the red blood-corpuscles and in the tissues of the spleen, liver, and brain, but not of the lungs.

(*f*) *Bile-pigment=bilirubin=hematoidin:* insoluble in water, ether, and alcohol; occurs as yellowish granules and masses which are often greenish if old; is found in jaundice.

2. **Autochthonous pigments**, formed by cells from colorless elements of nutrition. They all occur microscopically as lighter or darker brown granules; are insoluble in water, alcohol, dilute caustic potash, etc., and contain no iron.

Many of them are combined with fat, and hence seem to give some of the reactions for fat. They are found in the iris, retina, skin, ganglion-cells, Addison's disease, melanotic sarcomata, etc.

3. **Extraneous pigments**, entering the body from without. The most common examples are carbon in anthracosis pulmonum, iron in siderosis pulmonum, silver in argyria.

Pigments are recognized microscopically, partly by their color and form, partly by their chemical reactions, and partly, though less accurately, by the lesions or pathologic processes in connection with which they occur. They usually show best in contrast to red nuclear stains, such as alum or lithium carmine, but alum-hematoxylin often gives excellent results.

The pigments of the second and third groups are perfectly preserved by all the ordinary fixatives, of which alcohol, corrosive sublimate, and Zenker's fluid can be particularly recommended. Of the first group, melanin and hematoidin are preserved in any fixative. Hemoglobin and methemoglobin must be fixed in the solutions recommended for red blood-globules—namely, Zenker's fluid, corrosive sublimate, and Müller's fluid. Parhemoglobin and hemosiderin should be preserved in alcohol. Bile-pigment is turned green, according to Ziegler, by fixation in corrosive sublimate, and is thereby rendered more prominent. In alcohol it preserves its yellow color.

Carbon may be distinguished from melanin or any of the other pigments by the fact that it is insoluble in concentrated sulphuric acid. The only pigment for which micro-chemical color reactions are generally employed is hemosiderin, which really represents a group of pigments containing iron instead of one definite compound. Most of them will show the iron reaction after a shorter or longer time, but others, like the hemoglobin from which they are all derived, refuse to give it.

The iron compounds present are usually ferric salts, but occasionally ferrous. Both groups react in the same way to sulphate of ammonium, but only the ferric salts react to

ferrocyanid of potassium. For the ferrous salts, which occur much more rarely, the ferricyanid of potassium must be used. Dr. E. S. Wood suggests that a mixture of ferro- and ferricyanid of potassium be employed, so as to demonstrate at once both groups of iron compounds, as with the sulphate-of-ammonium method. In performing the iron reactions steel needles must be avoided.

Reactions for Iron in Hemosiderin.—Tissues should be hardened in alcohol or formaldehyde. I. Reaction for ferric salts with ferrocyanid of potassium and hydrochloric acid.

A. 1. Place sections for five to twenty minutes or longer in a 2 per cent. aqueous solution of ferrocyanid of potassium.

2. Transfer to acid alcohol (HCl 1 c.c. to 70 per cent. alcohol 100 c.c.) for five to ten minutes, or to glycerin plus $\frac{1}{2}$ per cent. hydrochloric acid. The iron appears bright blue in color.

If desired, the sections can be washed out after the acid alcohol, and passed through alcohol and oil to balsam. After the iron reaction has been performed, the nuclei may be stained in either alum or lithium carmine, or a little ferrocyanid of potassium may be added to the lithium carmine, and the reaction and nuclear stain thus effected by the same step.

B. H. Stieda's Method for Permanent Mounts with Nuclear Stains.—1. Stain several hours in lithium carmine.

2. Wash off quickly in water.

3. Place from four to six hours in a 2 per cent. aqueous solution of ferrocyanid of potassium.

4. Transfer to acid alcohol for six to twelve hours.

5. Wash quickly in water.

6. Alcohol, oil, Canada balsam.

II. The reaction for the ferrous salts is performed in the same way as for ferric salts, with the exception that ferricyanid of potassium is used instead of the ferro- compound.

III. *Reactions for Ferric and Ferrous Salts.*—*A.* Use a mixture of ferro- and ferricyanid of potassium (1 gram each to 100 c.c. of water), followed by acid alcohol. Nuclear stains and permanent mounts may be made as above.

B. 1. Place sections in a freshly prepared solution of sulphate of ammonium for five to twenty minutes, until they are dark or black-green in color.

2. Wash quickly in water.

3. Examine in glycerin or pass through alcohol and oil to xylol balsam.

The iron appears in the form of black or dark-greenish granules. Sulphate of ammonium causes similar precipitates with other metal salts, such as the nitrates of silver, lead, and mercury.

Petrifaction.—Calcification, the more common form of petrifaction, is the term applied to the infiltration of tissues with phosphate and carbonate of calcium. The salts appear microscopically as small, very refractive granules which may be mistaken for fat, or as large masses due to the fusion of granules. They are dissolved by hydrochloric or nitric acid (5 per cent. solution). If carbonate of lime is present, bubbles of carbon dioxid are set free. Phosphate of lime dissolves without effervescence. To differentiate between lime-salts and other substances soluble in hydrochloric acid use concentrated sulphuric acid to form sulphate of lime (gypsum), which appears as fine, short, radiating needles. On dissolving out the lime-salts a matrix of dead tissue or of hyaline material will usually be found left behind. As a rule, this hyaline material stains deep blue in alum-hematoxylin or red in Van Gieson's mixture.

The deposits of calcium salts themselves also stain with hematoxylin, so that it can be used to demonstrate the masses and coarser granules of them. The tissue must, however, first be freed of certain iron combinations, which are often associated with deposits of lime and also stain with hematoxylin. The following method is recommended by Roehl:

1. Fix in alcohol or formaldehyde.

2. Place sections in a half-concentrated solution of oxalic acid for fifteen to thirty minutes to remove the iron.

3. Wash thoroughly in water.

4. Stain in a 1 per cent. aqueous solution of hematoxylin

(which must be neither too fresh nor too old) for five to ten minutes.

5. Differentiate in water, to which a few drops of ammonia water are added, until the section is colorless and only the lime deposits remain stained.

6. Wash in water.

7. Counterstain with safranin. Alcohol; xylol; balsam. Lime-salts deep violet; nuclei red.

Von Kossa has shown that phosphate of calcium can be demonstrated by means of nitrate of silver, which forms silver phosphate on the surface of the granules and blackens in the presence of light. It gives an exaggerated picture of the amount of lime-salts present. Klotz has shown that the nitrate of silver acting for many hours affects calcium carbonate also: the granules become coated with silver carbonate, which in sunlight gives off carbon dioxid, leaving the black silver oxid. This process can be hastened by putting the sections, after staining and thorough washing, into a dilute soluble sulphide.

Von Kossa's Method.—1. Fix in alcohol, formaldehyde, or corrosive sublimate.

2. Place sections (frozen, celloidin, paraffin) in a 1 to 5 per cent. aqueous solution of nitrate of silver for thirty to sixty minutes (von Kossa), three to twelve hours (Klotz).

3. Wash thoroughly in distilled water.

4. Mount in glycerin or, after dehydration and clearing, in xylol balsam.

The lime is stained deep black. The nuclei can be counterstained with alum carmine or safranin after the silver staining.

Another form of petrifaction is that found in gout, due to the infiltration of certain tissues with uric-acid salts, of which urate of sodium is the most common. The crystals are soluble with difficulty in cold water, insoluble in alcohol and ether. Therefore, to study the deposits in connection with the lesions, fix in 95 per cent. alcohol and imbed in celloidin; stain sections quickly in a cold solution of alum-hematoxylin. Wash quickly in cold water and transfer to alcohol. Clear and mount in balsam.

THE STAINING OF BACTERIA IN TISSUES.

Bacteria are demonstrated in sections of tissues almost entirely by means of the aniline dyes, of which three have thus far proved themselves to be particularly valuable—namely, methylene-blue, methyl-violet, and fuchsin. These dyes are employed in aqueous or dilute alcoholic solutions, of which the effective staining power is greatly increased by means of heat and by the addition to the solutions of certain chemical substances.

The effect of moderate heat is obtained by placing the sections in the incubator for several hours, or greater heat for a short time is utilized by warming the staining solution on the slide over a small flame for a few seconds or minutes, keeping the fluid steaming, but not allowing it to boil.

Of the various methods employed to increase the staining power of aniline dyes by means of chemical substances, the most successful have been the use of caustic potash with methylene-blue, of aniline oil with methyl-violet and fuchsin, and of carbolic acid with fuchsin and methylene-blue.

For decolorizing sections after they have been stained, the most commonly employed reagents are—

1. Acetic acid in dilute aqueous solutions, 1 : 100, 1 : 1000.
2. Alcohol.
3. Iodin in iodid-of-potash solution (with certain dyes only).
4. Mineral acids in various strengths.
5. Chlorid of aniline.
6. Acid aniline colors added to the alcohol to increase its extractive power.
7. Aniline and ethereal oils.

The choice of a decolorizer varies with the staining solution employed and with the organism that is to be stained.

Sections which are to be stained for bacteria may be divided into two classes:

1. Sections free from celloidin, subdivided into—
    a. Sections cut without an imbedding mass;
    b. Paraffin sections;
    c. Sections from which the celloidin has been removed.

2. Sections infiltrated with celloidin.

Celloidin imbedding is to some extent a drawback to the stains for certain organisms, because the celloidin tends to hold the color, so that the bacteria are not so distinct as they otherwise would be. Still, it is so important to be able to stain bacteria in celloidin sections that particular care is devoted in the following pages to methods which obviate most of the difficulties.

Paraffin sections should, as a rule, be attached to the slide by means of Mayer's glycerin-albumin mixture.

It will usually be found advisable to attach celloidin sections to the slide by means of ether-vapor. They will then keep perfectly flat in any staining solution, and may be heated without danger of wrinkling or contracting. The heat should never be applied directly under a section, but at one end of the slide.

All bacteria yet known will stain when placed in appropriate staining solutions. Some, however, are stained quickly, while others are stained with difficulty; some give up the stain readily to decolorizers, while others retain it tenaciously. In consequence of their reactions to certain dyes and to certain decolorizers, bacteria, from the point of view of staining, may be divided into three groups:

1. Bacteria which do not stain by Gram;
2. Bacteria which stain by Gram;
3. Bacteria which stain by the tubercle bacillus method.

Two at least of the organisms in the third group will also stain by Gram. The organisms of the second and third groups are much more easily demonstrated in tissues than those in the first group, because it is possible to stain them of one color and the nuclei of the cells of another color. In other words, it is possible to stain them so that they are differentiated from the tissue in which they lie, and hence stand out prominently.

The organisms of the first group have no differential stain; they take the same color as the nuclei of the tissue. Moreover, although they stain easily, most of them do not stain deeply, and readily part with the color they have taken up.

Pathogenic Bacteria which do not Stain by Gram.

(See page 95.)

Of these organisms certain ones deserve special mention on account of their frequent occurrence or on account of the difficulty of demonstrating them in tissues, and certain variations in staining methods which have proved serviceable will be given. Löffler's methylene-blue solution is generally considered the most useful stain for this class of bacteria, but excellent results can also be obtained with Unna's alkaline methylene-blue solution preceded by eosin, especially after fixation in Zenker's fluid. Sometimes it is advisable to stain deeper than usual with the methylene-blue, to dehydrate quickly in absolute alcohol so as to extract as little color as possible, and to clear in xylol and mount in balsam in the usual way. The mounted sections are then placed in direct sunlight until faded to the right degree to show the bacteria deeply stained.

Löffler's Methylene-blue Stain for Bacteria.—1. Stain paraffin sections twenty minutes to twenty-four hours.

2. Wash in weak acetic acid, 1 : 1000, for ten to twenty seconds.

3. Absolute alcohol, two or three changes, to differentiate and dehydrate (as a rule, only a few seconds are required for this step).

4. Xylol.

5. Xylol balsam.

For celloidin sections use 95 per cent. alcohol; blot, and treat with xylol; repeat until sections are clear; mount in xylol balsam.

This solution of methylene-blue is extremely useful, because it will stain all bacteria except the tubercle-bacillus group. Other solutions which may be used in the same way are—aniline-methyl-violet, Stirling's solution of methyl-violet, simple aqueous solutions of methyl-violet, and Ziehl's carbol-fuchsin.

Methyl-green-pyronin Stain (Unna-Pappenheim) as modified by Saathoff for Bacteria:

Methyl-green,	0.15;
Pyronin,	0.50;
96 per cent. alcohol,	5.00;
Glycerin,	20.00;
2 per cent. carbol-water ad	100.00.

Stain sections two to four minutes, then wash in water, dehydrate quickly in absolute alcohol, clear in xylol, and mount in balsam. If acetone is used instead of absolute alcohol for dehydration there is less danger of decolorizing the cystoplasm of the cells.

Gonococcus.—Löffler's solution gives good results.

Touton recommends staining sections in carbol-fuchsin and washing out in alcohol.

Typhoid Bacillus.—Typhoid bacilli in stained sections are generally best hunted for with a low power. The characteristic colonies which they form are easily recognized. Good results in staining can be obtained with Löffler's methylene-blue solution used in the manner already described, but the stain is never very intense. For rendering the bacilli rather more prominent, so that small groups of them may be recognized, Flexner has advised the two following methods:

A.—1. Stain paraffin sections in Löffler's methylene-blue solution for two hours.

2. Acetic-acid solution, 1 : 1000, for several minutes.

3. Dehydrate in absolute alcohol.

4. Oil of cloves to clear and differentiate.

5. Xylol, several changes.

6. Xylol balsam.

B.—1. Stain sections in Stirling's gentian-violet solution for ten minutes.

2. Acetic-acid solution, 1 : 1000, for some minutes.

3. Dehydrate quickly in 95 per cent. alcohol.

4. Transfer to slide, blot, add oil of cloves to clear, and differentiate. Change the oil several times until the desired differentiation is obtained.

5. Wash off section several times with xylol.

6. Xylol balsam.

Influenza Bacillus.—1. Harden in alcohol.

2. Stain half an hour or more in carbol-fuchsin diluted with 20 parts of water.

3. Wash out in a watch-glass of water to which is added a drop of glacial acetic acid until the section appears gray-violet in color.

4. Alcohol, xylol, balsam.

Glanders Bacillus.—The bacilli are usually not numerous, and are scattered about in a mass of deeply staining fragmented nuclei, so that often they are recognized with great difficulty. The ordinary eosin-methylene-blue stain, after fixation in Zenker's fluid, can be highly recommended for demonstrating them. The following special methods have long been used for the same purpose.

Löffler's Methylene-blue Stain for Sections.—1. Stain paraffin sections twenty minutes in Löffler's methylene-blue solution or in equal parts of aniline-methyl-violet and 1 : 10,000 KOH solution.

2. Place for five seconds in the following solution:

Distilled water,	10 c.c.;
Concentrated sulphuric acid,	2 drops;
5 per cent. oxalic acid,	1 drop.

3. Wash out quickly in distilled water.
4. Absolute alcohol.
5. Xylol.
6. Xylol balsam.

It is recommended to place the section for a few minutes before staining in the 1 : 10,000 caustic-potash solution.

Schutz's Method.—1. Stain twenty-four hours in equal parts of concentrated alcoholic solution of methylene-blue and caustic potash, 1 : 10,000.

2. Wash in acidified water.
3. 50 per cent. alcohol for five minutes.
4. Absolute alcohol for five minutes.
5. Xylol.
6. Xylol balsam.

Noniewicz's Method.—1. Stain in Löffler's methylene-blue solution two to five minutes.

2. Wash in water.
3. Decolorize one to five seconds in

$\frac{1}{2}$ per cent. acetic acid,	75 parts;
$\frac{1}{2}$ per cent. aqueous solution of tropeolin,	25 "

4. Wash in water.
5. Dehydrate section on slide with filter-paper; then in the air; finally, over small flame.
6. Clear by dropping xylol on it repeatedly.
7. Xylol balsam.

Chancroid Bacillus.—The methods given for the glanders bacillus are also useful for this bacillus.

Friedländer's Capsule-bacillus. — The following method is recommended for staining the capsules in sections:

1. Stain for twenty-four hours in the incubator in the following solution:

Concentrated alcoholic solution of gentian-violet,	50;
Distilled water,	100;
Glacial acetic acid,	10.

2. Wash out in a 1 per cent. solution of acetic acid.
3. Alcohol.
4. Oil.
5. Xylol balsam.

If the process of decolorization is stopped at the right moment, the capsules will be pale blue, while the bacilli will be stained deep blue.

Pathogenic Bacteria which Stain by Gram.

(See page 96.)

These organisms, with the exception of the tubercle-bacillus group, are all readily stained by the general methods employed for staining under Group 1. For staining most of them in sections, however, the differential Gram-Weigert method will be found to give the most satisfactory results.

The Gram Staining Method.—Directions for staining paraffin sections: 1. Stain in aniline-methyl-violet five to twenty minutes.

2. Wash in normal salt solution or water.

3. Iodin solution (1 : 2 : 300) one minute.

4. Wash in water.

5. Absolute alcohol, several changes, until no more color is given off and the section is apparently decolorized.

6. Xylol.

7. Xylol balsam.

This method is not suited for celloidin sections, because the alcohol does not decolorize the celloidin sufficiently. In fact, it is better to reserve Gram's method for cover-slip work alone, and to use instead of it, for sections of all kinds, Weigert's modification. This consists simply in the use of aniline oil instead of alcohol as a decolorizer. The method is easily acquired, is perfectly adapted to celloidin sections, and the results are more perfect than after Gram.

The Gram-Weigert Staining Method.—Directions for staining celloidin sections: 1. Stain sections with lithium carmine in the ordinary way (see page 326).

2. After dehydrating in 95 per cent. alcohol stick the section to the slide with ether-vapor.

3. Stain in aniline-methyl-violet five to twenty minutes.

4. Wash off excess of stain in normal salt solution.

5. Iodin solution (1 : 2 : 100) one minute.

6. Wash off in water.

7. Blot section with filter-paper to remove as much of the moisture as possible.

8. Aniline oil, several changes, to dehydrate and to remove all excess of color.

9. Xylol, several changes to remove the aniline oil completely.

10. Xylol balsam.

Bacillus of Rhinoscleroma.—Method of staining capsules in sections of tissues hardened in alcohol (Wolkowitsch): 1. Stain twenty-four to forty-eight hours in aniline-methyl-violet.

2. Wash off in water.

3. Iodin solution one to four minutes.

4. Absolute alcohol.

5. Oil of cloves, which removes still more of the color.
6. Xylol.
7. Xylol balsam.

According to Wolkowitsch, the hyaline masses in rhinoscleroma stain intensely with methyl-violet, gentian-violet, methylene-blue, and fuchsin; less with safranin, and not at all with hematoxylin. Eosin stains them well. Double staining with hematoxylin and eosin is therefore to be recommended highly.

Actinomyces.—In staining the actinomyces it is important to stain not only the filaments and other forms of the organism, but also the hyaline swollen sheaths which surround the ends of the filaments. Eosin followed by methylene-blue sometimes gives good results. Good preparations can also be obtained by staining in alum-hematoxylin, followed by a strong solution of eosin; place the sections for five to thirty seconds in acid alcohol, and then wash thoroughly in water before dehydrating in alcohol. It is believed that the two following methods will give better results than can be obtained by any of the methods previously published for this purpose. The first is, perhaps, the better and surer, although the clubs are sometimes brought out more intensely by the second method.

Formaldehyde and alcohol fixation are preferable to Zenker's fluid for the study of this micro-organism, but not for the study of the lesions produced by it.

Mallory's Stains.—Method No. 1.—1. Stain sections deeply in a saturated aqueous solution of eosin for at least ten minutes.

2. Wash off in water.
3. Stain in aniline-methyl-violet two to five minutes.
4. Wash off with normal salt solution.
5. Iodin solution (1 : 2 : 100) one minute.
6. Water. Blot with filter-paper.
7. Aniline oil until section is clear.
8. Xylol, several changes.
9. Xylol balsam.

A light preliminary stain with alum-hematoxylin will often be found useful to bring the nuclei out sharply.

Method No. 2.—1. Stain lightly in alum-hematoxylin three to five minutes.

2. Wash in water.
3. Dehydrate in 95 per cent. alcohol.
4. Fasten section to slide with ether-vapor.
5. Aniline-methyl-violet five to twenty minutes.
6. Wash off with water.
7. Dry with filter-paper.
8. Aniline saturated with fuchsin one to three minutes.
9. Wash out the fuchsin with pure aniline until the clubs are sharply differentiated: watch the process under the low power of the microscope.
10. Xylol, several changes.
11. Xylol balsam.

The polymorphous bacterium is stained blue, the swollen membrane (the club), light to dark pink. By these methods it is possible to demonstrate in sections containing young colonies the ends of the threads stained blue surrounded by the swollen cell-membrane stained pink.

Bacteria that Stain by the Tubercle Bacillus Method.

Tubercle bacillus;
Leprosy bacillus;
Smegma bacillus.

The important point about staining *tubercle bacilli* is to stain them deeply enough in the beginning; then there is little danger of their fading in the subsequent steps of contrast staining. It is probable that carbol-fuchsin, used hot, is the most powerful stain we have for this purpose. If the solution is steamed, generally on the slide, one to five minutes are probably sufficient for all purposes. Tubercle bacilli stain well, not only after alcohol, but also after most of the other fixing reagents, such as corrosive sublimate, Zenker's fluid, Flemming's solution, etc.

Ehrlich's Method.—1. Stain paraffin sections in aniline-fuchsin or methyl-violet for half an hour to twenty-four hours, or for one to five minutes if solution is heated to steaming.

2. Wash in water.

3. Decolorize in 20 per cent. nitric acid one-half to one minute.

4. Wash in 70 per cent. alcohol until no more color is given off.

5. Contrast-stain in a saturated aqueous solution of methylene-blue or of Bismarck brown one to two minutes.

6. Wash in water.

7. Dehydrate in absolute alcohol.

8. Xylol, xylol balsam.

Ziehl-Neelson-Gabbet Method.—1. Stain paraffin sections in carbol-fuchsin solution, warming the solution so that it steams one to three minutes.

2. Wash in water.

3. Decolorize and stain for contrast in sulphuric-acid-methylene-blue solution one minute (see page 298).

4. Wash in water.

5. Absolute alcohol

6. Xylol.

7. Xylol balsam.

This method is not suited to celloidin sections, because the celloidin retains too deep a blue stain.

Kühne's Method.—1. Stain paraffin sections lightly in alum-hematoxylin.

2. Wash in water.

3. Stain in carbol-fuchsin one to five minutes if warmed; longer if cold.

4. Wash in water.

5. Aniline hydrochlorate, 2 per cent. aqueous solution, fifteen seconds.

6. Wash in water.

7. Absolute alcohol.

8. Xylol.

9. Xylol balsam.

To Stain Tubercle Bacilli in Celloidin Sections.—1. Stain rather lightly in alum-hematoxylin.

2. Wash in water.

3. Dehydrate in 95 per cent. alcohol.

4. Attach sections to slide by the ether-vapor method.
5. Carbol-fuchsin two to five minutes steaming.
6. Water.
7. Orth's discharging fluid (acid alcohol) one-half to one minute.
8. Wash thoroughly in several changes of water to remove acid completely and to bring back blue color to nuclei.
9. Alcohol 95 per cent. until fuchsin is entirely discharged.
10. Aniline followed by xylol; or blot and treat with xylol.
11. Xylol balsam.

The advantages of this method are—that the celloidin is colorless; the nuclei are stained blue; the rest of the tissue is colorless; the tubercle bacilli stand out in sharp contrast. It is sometimes an advantage to bring out the cell-protoplasm and the intercellular substance by staining the sections, after decolorization in alcohol, in an aqueous solution of orange G or methyl-orange for a few seconds.

The **bacillus of leprosy** stains more easily than the tubercle bacillus. Simple aqueous solutions of the aniline dyes are sufficient. The same methods can be employed as for tubercle bacilli. A method recommended by Flexner will be found very useful.

1. Stain in alum-hematoxylin so as to get a sharp nuclear stain.
2. Wash in water.
3. Carbol-fuchsin two to five minutes steaming, or thirty to sixty minutes cold.
4. Water.
5. Treat on the slide with iodin solution one-half to one minute.
6. Water.
7. Blot; clear and differentiate in aniline oil.
8. Xylol; balsam.

Baumgarten gives the following differential stain for leprosy bacilli:

1. Stain six to seven minutes in a dilute solution of fuchsin (5 drops of a concentrated alcoholic solution to a watch-glass of water).

2. Discharge one-quarter minute in nitric-acid alcohol (nitric acid 1, alcohol 10).

3. Wash in water.

4. Contrast-stain in a saturated aqueous solution of methylene-blue.

5. Alcohol.

6. Xylol.

7. Balsam.

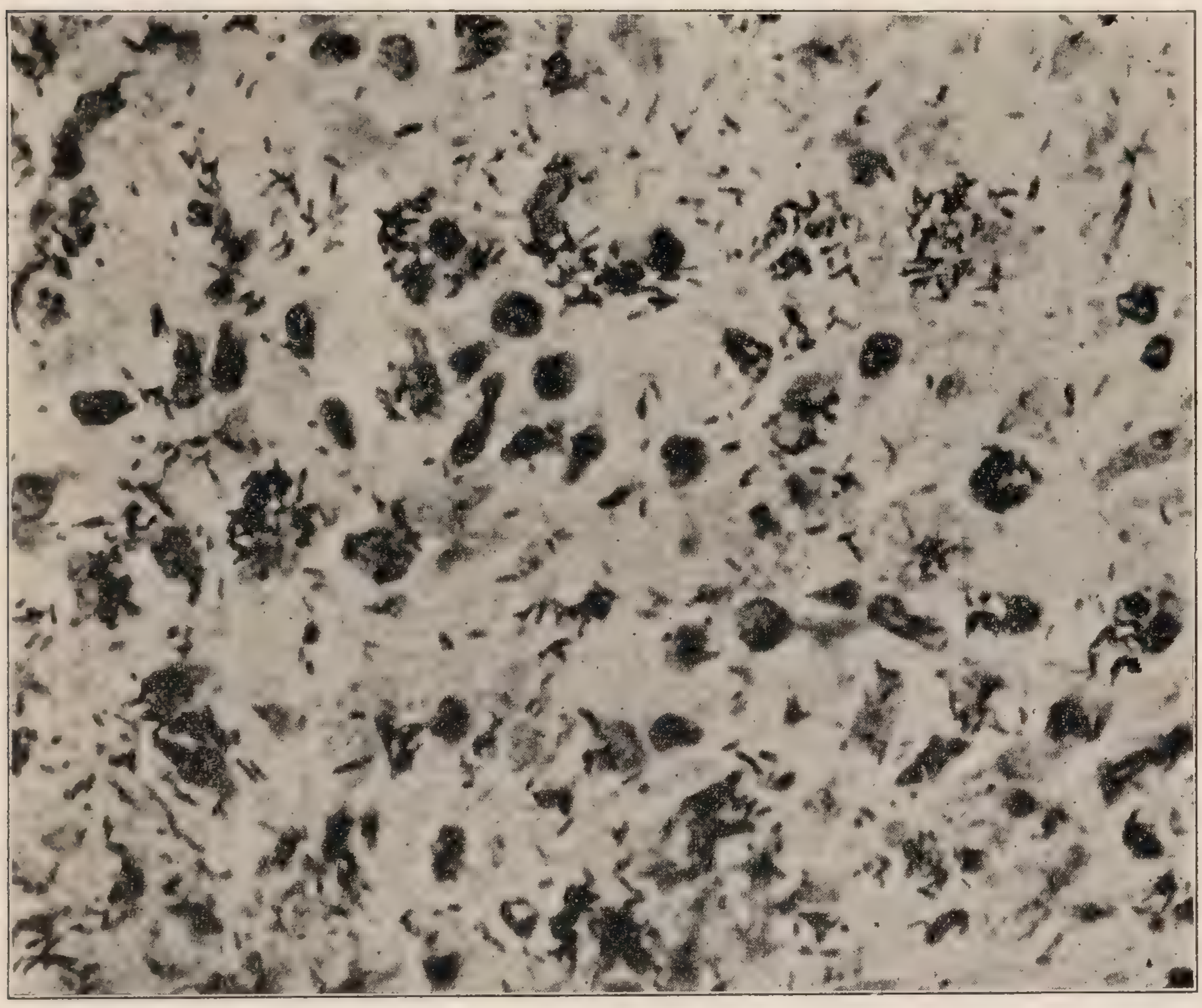


FIG. 129.—Bacillus of leprosy: section through a cutaneous nodule, showing the bacilli in the tissue; × 750 (Wright and Brown).

While leprosy bacilli stain readily by this method, tubercle bacilli will not stain in so short a time.

Syphilis.—Methods of Demonstrating Treponema Pallidum (Spirochæte Pallida) in Smear Preparations.—The lesions are to be cleansed from any adherent exudate. The smear preparations are to be made from the juice of the tissue obtained by pressure and scraping. An excess of blood should be avoided. The preparations are then dried in the air and may be stained by the following methods:

1. *Method with Wright's Blood Stain.*—Place in a test-tube 10 c.c. of distilled water, 1 c.c. of the blood-staining fluid, and 1 c.c. of a 0.1 per cent. solution of potassium carbonate. Heat to boiling and cover the preparation with the hot mixture. After three or four minutes, when the fluid on the preparation has become of a violet color and a thin yellow metallic scum has formed on the surface, pour off and again cover the preparation with the hot mixture after

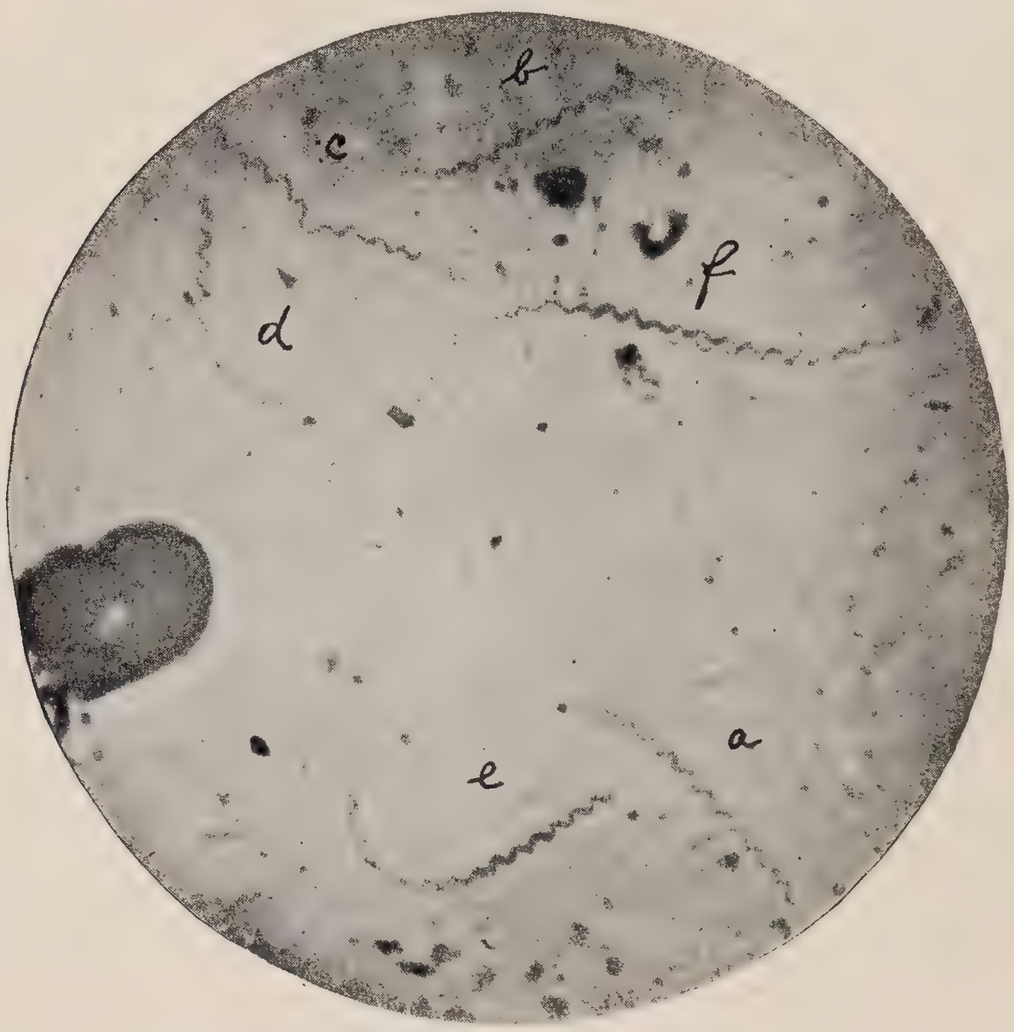


FIG. 130.—Treponema pallidum in smear preparation (Goldhorn).

again heating in the tube. Repeat this once more. Dry and mount.

The material should be thinly spread on a cover-glass, not on a slide, the cover-glass to be held level with forceps during the staining.

The treponemata should stain intensely violet.

2. *Giemsa's Method.*—This is the same as his method for staining malarial parasites (see page 448), except that the preparation is fixed in absolute alcohol for fifteen to twenty

minutes, and that to the water used for diluting the staining fluid 1 to 10 drops of a 0.1 per cent. solution of potassium carbonate is added. Preparations which are overstained may be differentiated by washing in distilled water for one to fifteen minutes.

Recently it has been shown that heating the diluted staining fluid on the preparation stains the treponemata much more intensely. This modified method is as follows:

Ten drops of Giemsa's staining fluid are mixed by gently shaking with 10 c.c. of distilled water immediately before proceeding to the staining. The preparation is fixed in absolute alcohol fifteen minutes, or by drawing three times through the flame. It is then covered with the diluted staining fluid and warmed until a slight steam arises over the flame, and allowed to cool about fifteen seconds, when the diluted staining fluid is poured off and replaced by fresh fluid, and this again warmed to steaming and allowed to cool for about fifteen seconds. This process is repeated four or five times, after which the preparation is washed, dried, and mounted in balsam. In this modified method the staining of the parasites is intensely dark red. It is important that the slide or cover-glass be free from grease, and that the test-tube and the cover-glass or slide forceps be clean, free from acid, and from any precipitated stain.

3. *Stern's Silver Method.*—The preparation, without special fixation, is placed in the incubator for some hours, then in a 10 per cent. solution of nitrate of silver in a colorless glass container, and is allowed to remain in diffuse daylight for some hours. When the preparation takes on a metallic lustre, the impregnation of the silver is said to be complete. The treponemata appear intensely black stained, as in sections prepared by Levaditi's method.

According to Flexner, better results are obtained with moderately thick smears or impression preparations and by exposure to weak, diffuse daylight from three to five days.

4. *Ghoreyeb's Method.*—In this method the following solutions are used:

1. One per cent. aqueous solution of osmic acid.

2. Liquor plumbi subacetatis, diluted one hundred times with distilled water. This diluted solution should be freshly prepared.

3. Ten per cent. aqueous solution of sodium sulphid. A thin smear is preferable. No heat fixation is necessary.

The smear is stained as follows:

1. Cover with osmic acid solution for thirty seconds.
2. Wash in water.
3. Cover with lead subacetate ten seconds.
4. Wash in water.
5. Cover with sodium sulphid solution ten seconds.
6. Wash in water.

This process is gone through with three times. Following this the osmic acid solution is applied for thirty seconds, and

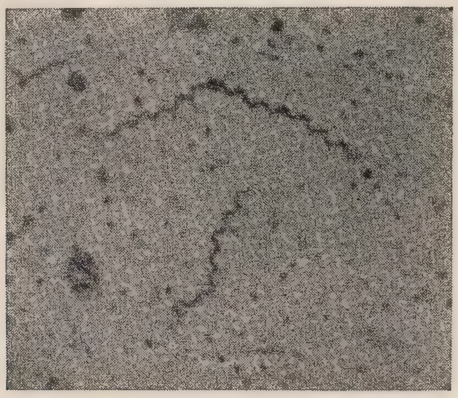


FIG. 131.—Treponema pallidum; smear preparation from a cutaneous papule; stained by Ghoreyeb's method; × 1500 (photo. by L. S. Brown).

the specimen is then washed in water, dried, and mounted in balsam. A thorough washing in running water is essential after the application of each solution to prevent the formation of excessive precipitates.

The osmic acid, the first time applied, acts as a fixative and a mordant. The lead unites with the albumin to form lead albuminate, a compound insoluble in water. The sodium sulphid transforms the lead albuminate into lead sulphid, and causes the preparation to become stained brown. The osmic acid turns the brown color to black. The spirochetes, bacteria, and cellular detritus are stained black.

5. *India Ink Method of Burri.*—Approximately equal parts of the juice from the lesion and of fluid India ink are quickly mixed together on a slide with the aid of a platinum loop,

spread thinly, and allowed to dry thereon. When dry, the preparation is ready to be examined directly with the oil-immersion objective without covering it with balsam and a cover-glass. If the material contains many cellular elements or detritus, it will be necessary to dilute the ink with water. The preparation should have a brown color. The spirochetes and bacteria appear as unstained bodies in a brown to black background.

Some specimens of fluid India ink are said to contain spirochete-like bodies, and therefore the ink used should be known to be free from such.

6. *Levaditi's Method for Staining Treponema Pallidum in Sections.*—1. Pieces of tissue about 1 mm. thick are placed in 10 per cent. formaldehyde for twenty-four hours.

2. Rinse in water and place in 95 per cent. alcohol for twenty-four hours.

3. Place in distilled water until the tissue sinks to the bottom of the container.

4. Place in a 1.5 or 3 per cent. solution of nitrate of silver and keep in the incubator at 38° C. for three to five days. The stronger solution of nitrate of silver is preferable for tissues removed during life.

5. Wash in distilled water and place in the following solution for twenty-four to seventy-two hours at room temperature:

Pyrogallic acid,	2–4 gm.;
Formaldehyde,	5 c.c.;
Distilled water,	100 c.c.

6. Wash in distilled water.

7. Dehydrate in alcohol, clear in chloroform, and embed in paraffin in the usual manner.

The treponemata are stained intensely black by the precipitation of metallic silver upon them. The reticulum stains brown, while the other elements of the tissue generally are of a yellow color. The sections may be counterstained with some aniline dye, but this is of doubtful advantage.

7. *Eyenes and Sternberg's Method for Staining Treponema Pallidum in Sections.*—1. Fix well in 10 per cent. formalin. Cut sections, by freezing or in celloidin, as thin as possible.

2. Wash the sections in distilled water, and place them in a 1 per cent. aqueous solution of silver nitrate for thirty to thirty-five minutes in the incubator or longer at room temperature in the dark. The period of time in the incubator is important.

3. Place the sections in the following mixture until they are dark brown (one to two minutes), removing them before a precipitate occurs:

2.5 per cent. aqueous solution of siver nitrate,	10 c.c.;
10 per cent. aqueous solution of gelatin (warm),	10 "
50 per cent. aqueous solution of gum arabic,	10 "
5 per cent. aqueous solution of hydroquinone,	5 "

The ingredients are to be well mixed both before and after the addition of the hydroquinone, which should be added last.

4. Wash for one to two minutes in 10 per cent. aqueous solution of sodium hyposulphite.

5. Wash in water, dehydrate, clear, and mount.

The microscopical appearances are essentially the same as those produced by Levaditi's method.

METHODS OF EXAMINATION OF ANIMAL PARASITES.

Malarial Organisms.[1]—Three varieties of the plasmodium malariæ have been described—namely, the tertian, quartan, and estivo-autumnal parasites. They develop within or upon the red corpuscles and cause the destruction of the corpuscles affected. The earliest forms of the parasite appear in the blood during the latter part of the malarial paroxysm or shortly after it. At this time they appear as small, color-

[1] For some important details, here omitted, concerning the morphology and biology of the malarial parasites the reader is referred to the authoritative papers by Mary Rowley-Lawson, *Jour. Exp. Med.*, xiii., p. 263, and by Charles F. Craig, Osler's *Modern Medicine*, i., p. 392.

less, disc-shaped hyaline bodies which occupy but a small portion of the blood-corpuscles. They possess a varying degree of ameboid movement, the amount depending upon the type of the organism. These ameboid movements are best observed on the warm stage. During the process of development the parasites increase in size and more or less completely fill the red corpuscles containing them. Small particles of reddish-brown pigment are produced, during their growth, from the hemoglobin of the corpuscles in which the organisms are developing. These granules show varying degrees of motion, probably imparted to them by the movements of the parasites. At first the pigment appears to be scattered about in the corpuscle, but it is in reality in the extremities of the pseudopodia. Later it appears more evenly spread about in the periphery. Toward the end of the cycle of development the pigment collects in the center of the parasite; at this time the ameboid movements have ceased, indications of segmentation occur, and the parasite nearly or completely fills the corpuscle. Oftentimes at this stage only a small portion of the corpuscle is visible at some point on the edge of the parasite.

The beginning of segmentation is indicated by a number of radial lines extending from the periphery of the parasite toward the central clump of pigment. Segmentation takes place, and the pigment is surrounded by a number of distinct segments which vary with the type of the organism. Each of these segments shows a central refractive spot which probably is the nucleus. At this time one notices small hyaline bodies, like those of the early stage in the development of the parasite, in some of the red blood-corpuscles. Oftentimes such a regular process of segmentation is not observed, but enough has been said to indicate the manner in which reproduction occurs. Segmentation is the indication of an approach of a paroxysm. Extra-cellular forms of the parasites are not infrequently seen. They may be fully grown organisms which have destroyed the corpuscles that contained them, or they may be partly grown organisms which have left the corpuscles. These free para-

sites are indistinct in outline and contain pigment. They possess ameboid movements, and may be considerably larger than a red blood-corpuscle. Various changes are observed in them:

1. They may increase in size until they become nearly as large as polymorphonuclear leucocytes. With the increase in size there is a gradual cessation in the movement of the pigment-granules, until finally the organisms present the appearance of misshapen masses of protoplasm containing motionless pigment-granules.

2. They may undergo fragmentation and give off several small circular pigmented bodies.

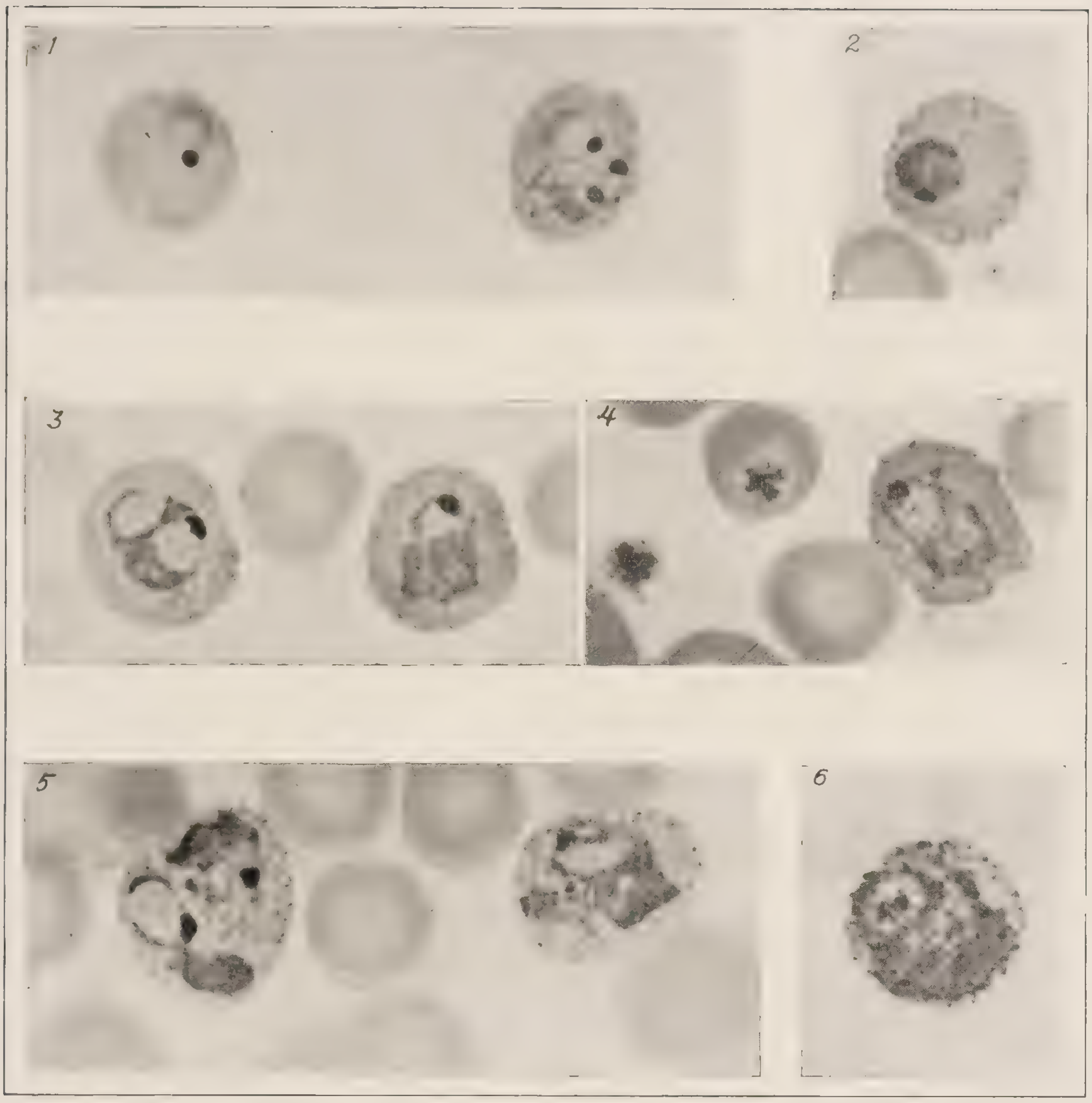
3. Vacuolization may occur.

4. Flagellate forms may develop. One or more thread-like processes are thrust out from the organisms. These flagella may contain pigment, and may break away from the organism and move about among the corpuscles, looking not unlike the spirilla of relapsing fever.

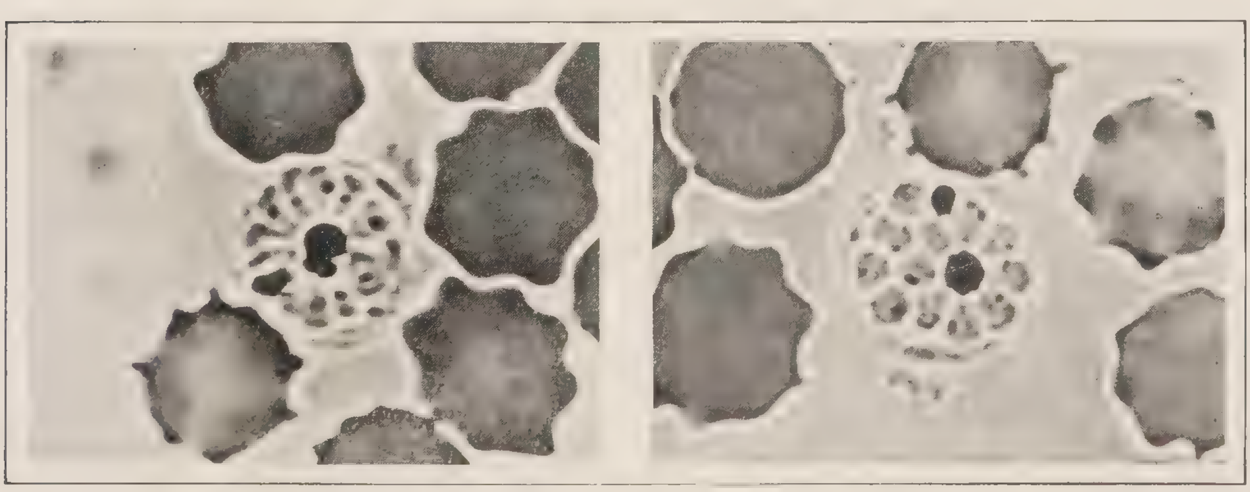
The three varieties of parasites differ from one another in a number of ways. The chief differences are the length of the cycle of development; the size of the full-grown organisms; the difference in the refractibility of the organisms; the quantity, size, and color of the pigment-granules; the degree of ameboid movement; and the number and shape of the segments into which the full-grown organisms divide. In the earliest stage the varieties or organisms cannot be distinguished from each other.

The tertian parasite completes its cycle of development in about forty-eight hours. When it has attained its fullest growth it almost fills the corpuscle, which has become larger than normal. This organism is less refractive than either of the other two. The pigment-granules are more numerous, finer, and more reddish-brown in color; the ameboid movements are much more active; the segments are more irregular in shape and more numerous than those of the quartan parasite, varying from twelve to twenty in number.

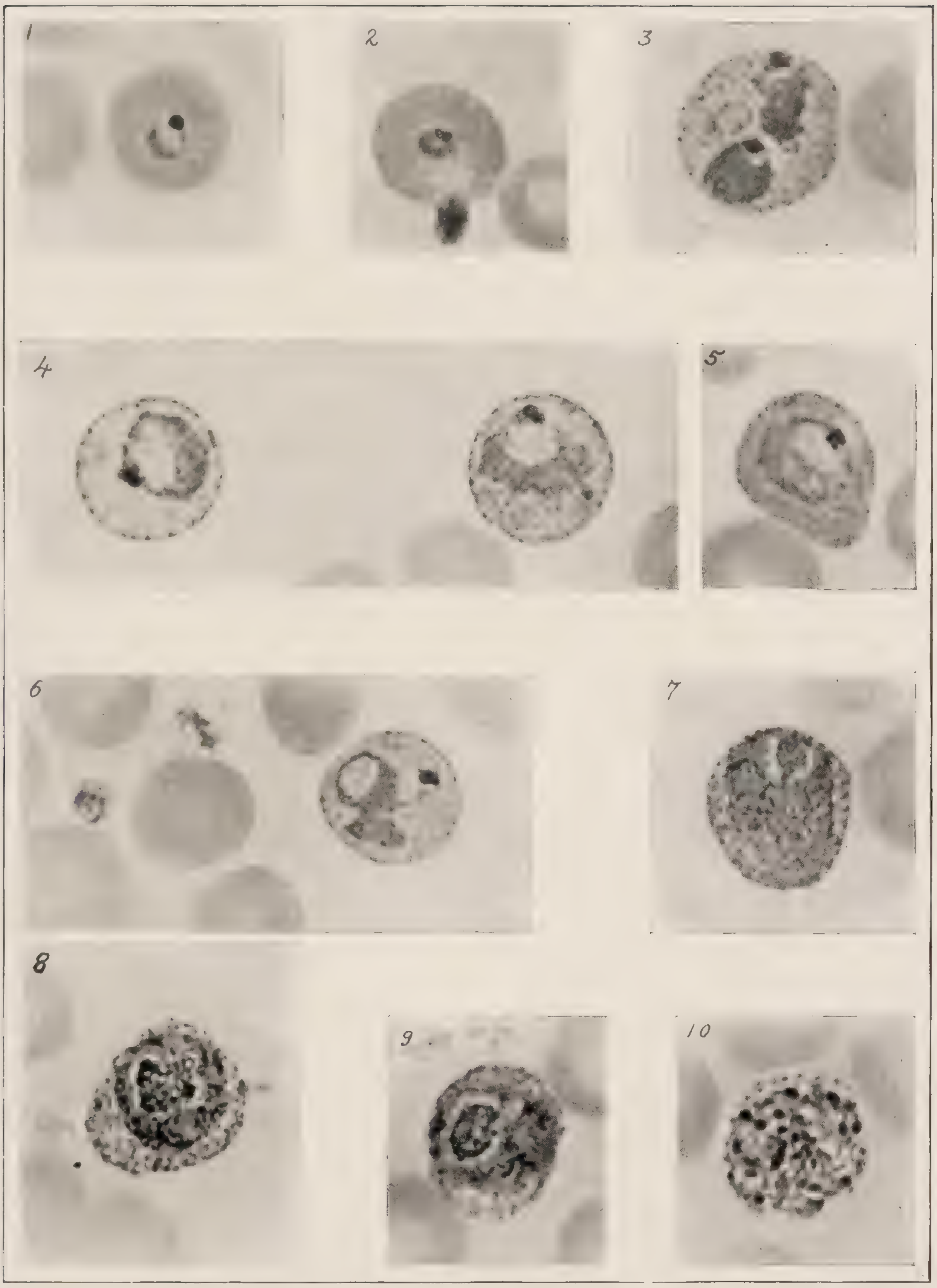
The quartan appears to complete its cycle of development in from sixty-four to seventy-two hours. The full-grown



Tertian malarial parasites in red blood-corpuscles (Wright's stain): 1, Young parasites (in the corpuscle on the right two or three parasites); 2, young parasites; 3, half-grown parasites; 4, half-grown parasite (on the left, a blood-plate and near the center another blood-plate lying on a red corpuscle); 5, half-grown parasites (in the corpuscle on the left two parasites); 6, full-grown parasite (the nucleus lies in a clear space). All the infected blood-corpuscles in the foregoing figures contain minute granules that stain red (granular degeneration) (photos by L. S. Brown).



Two stages in the process of segmentation of a tertian malarial parasite in a red blood-corpuscle. Preparation of fresh blood, not stained (Wright and Brown).



Tertian malarial parasites in red blood corpuscles (Wright's stain) : 1, Young parasite ; 2, young parasite with blood plate at the margin of the corpuscle ; 3, two young parasites in one corpuscle ; 4 and 5, immature parasites ; 6, immature parasite (on the left two blood plates) ; 7, adult parasite (chromatin of nucleus in clear space) ; 8 and 9, adult parasites (the chromatin is the reticular mass near the center of the parasites) ; 10, segmenting parasite (the chromatin has divided into a number of separate dark colored masses. In the center some pigment). In all of the foregoing figures, except Figure 1, the granular degeneration of the infected red corpuscles is shown (photos by L. S. Brown).

organism does not fill completely the corpuscle, and the latter is not increased in size. The organism is more refractive than the tertian parasite. The pigment-granules are fewer in number, coarser, and have a darker-red color. The ameboid movements are slower; the segments are pear-shaped,

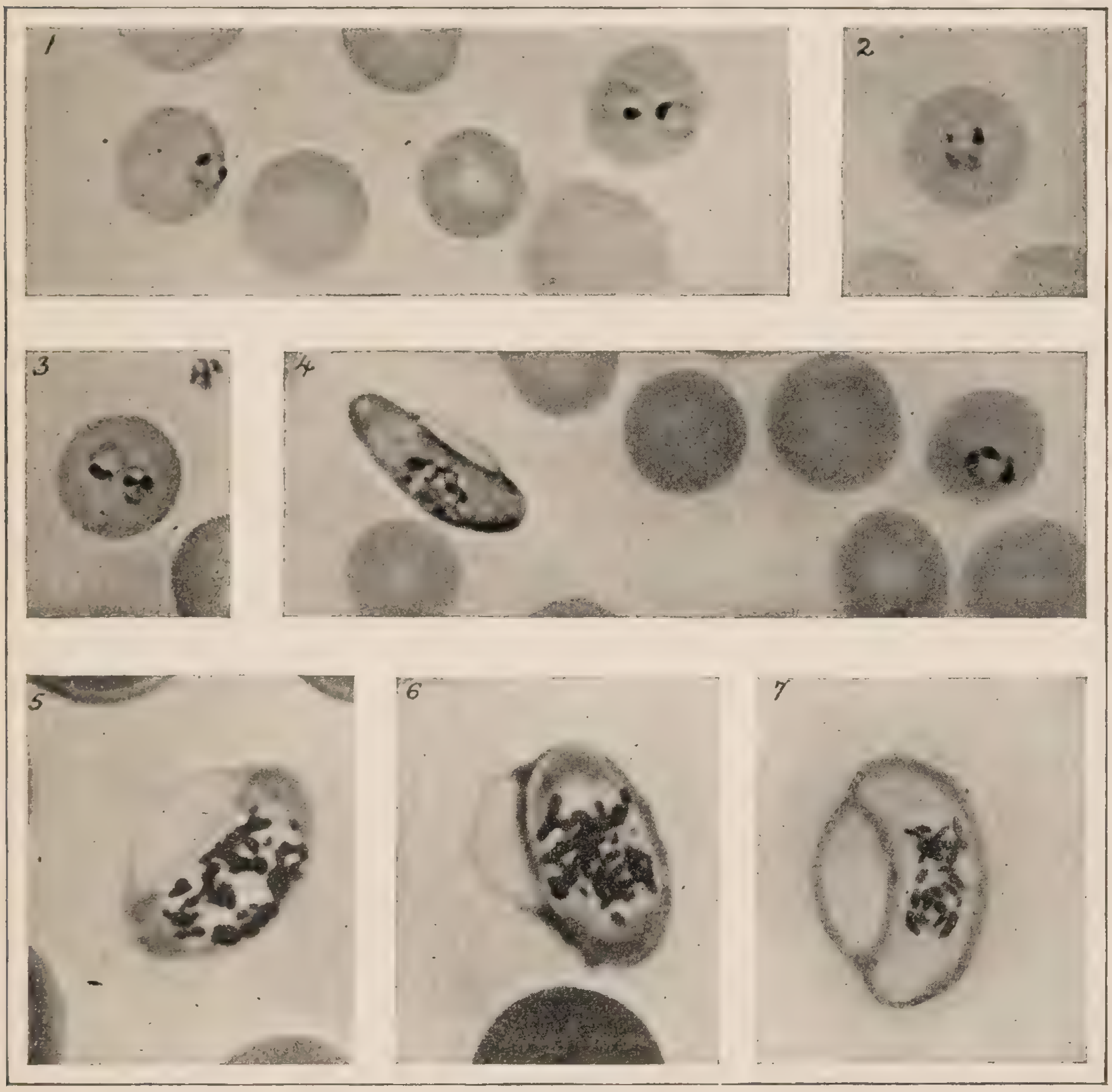


FIG. 132.—Estivo-autumnal malarial parasites in red blood-corpuscles (1, 2, 3, and 4, Wright's stain): 1, 2, and 4, young parasites; 3, on the left, a "crescent," on the concave side of which is shown a portion of the periphery of the red corpuscle, which it distends; on the right, a young parasite; 5 and 7, "crescents" in red blood-corpuscles; 6, ovoid form of parasite in a red blood-corpuscle (photos by L. S. Brown).

more symmetrical, and less numerous than those of the tertian parasite, varying from six to twelve in number. Segmenting organisms are more numerous in the peripheral circulation than in the case of the tertian parasite.

The estivo-autumnal parasite cannot be studied so thor-

oughly in the peripheral circulation, because the latter development and segmentation take place in the internal organs. The length of time required to complete its cycle of development is not so definitely settled. It appears to require from twelve to twenty-four hours, more or less. The full-grown organism is smaller than the tertian parasite, and the corpuscle which contains it is often smaller than normal and more or less distorted. The parasite is quite refractive. The pigment-granules are few in number and coarse. The ameboid movements are slow. After the duration of fever for from five days to a week or more, elongated, ovoid, or crescent-shaped bodies make their appearance. They are sometimes as large or larger than a red corpuscle. These bodies are not a result of segmentation, but appear to be a further development of the round hyaline bodies. They are highly refractive and contain granules of coarse pigment in the center. They lie usually at one side of the red corpuscles, the latter more or less completely filling the concavity between the two horns of the crescent. They may lie in the center of the corpuscles. Some of the apparently free ovoid bodies are turned in such a way as to present a convex surface toward the observer.

Double infections occur quite frequently in both tertian and quartan fever, and in the latter not infrequently triple infections occur. In the double infections two groups of parasites reach maturity on successive days and cause daily febrile paroxysms. In the triple infection of quartan fever three groups of organisms mature on successive days and cause corresponding paroxysms.

Methods of Examining the Blood for Malarial Organisms. —The organisms of malaria can be detected in fresh specimens of blood or in specimens of blood which have been fixed and stained.

In doubtful cases the parasites are more surely and easily found in cover-glass preparations of the blood fixed and stained by special methods.

NOTE.—The description of the development of the parasites is abstracted from Thayer and Hewetson's *The Malarial Fevers of Baltimore.*

The method employed in making cover-glass preparations of the blood has been thoroughly described (see preparation of cover-glass specimens in the Examination of the Blood, page 379).

Wright's stain for malarial parasites is identical with his blood-stain and is applied in the same way (see page 382). It gives the so-called Romanowsky stain to the parasites.

With the stain, the body of a malarial parasite stains blue, while the color of the chromatin varies from a lilac color through varying shades of red to almost black. In the young forms of the tertian and estivo-autumnal parasites the chromatin appears as a spherical, very dark-red body, while in the older forms of the tertian parasite it has a more lilac or purplish-red color, and may appear in the form of a reticulum. In the intermediate forms the color of the chromatin may present variations between these extremes (see Fig. 132).

Blood plates apparently situated within red blood-corpuscles may be mistaken by the inexperienced for young malarial parasites. This ought never to occur if one bears in mind the fact that the young parasite of all the three kinds should present by this method a dark-red, spherical nucleus and a homogeneous blue cytoplasm which is usually in the form of a definite ring (see Fig. 132).

Various workers have shown by their modifications of the Romanowsky method that red blood-corpuscles harboring malarial parasites have dark-red staining granules. These granules may be brought out by the present method, but in order to bring them out, it may be necessary to allow the staining fluid, after the addition of the water to it, to remain on the preparation for at least five minutes, and then not to decolorize or differentiate with water for as long a time or to such an extent as for ordinary blood preparations.

In examining a fresh specimen of the blood for the malarial organisms a glass slide is substituted for one of the cover-glasses, and the cover-glass which has the drop of blood on its surface is dropped lightly upon the glass slide and allowed to remain there. The first four or five drops of blood

should be quickly wiped away from the ear until a very small drop is obtained. Great care must be exercised to touch only the tip of the drop with the cover-glass, so as to avoid smearing the blood. If the blood is smeared on the cover-glass, the edges of the blood-drop will dry before the cover-glass can be transferred to the slide, and the blood will not spread. It is necessary that the blood should spread in a thin layer in order to study satisfactorily the individual corpuscles. If one desires to study the preparation for several hours, the edges of the cover-glass can be surrounded by melted paraffin or vaselin to exclude the air. The examination should be made with an oil-immersion lens. It should be remembered that the action of cold inhibits the ameboid movements of the parasites; it may be necessary, therefore, at times to warm the slide before examining the specimen. Evaporation not infrequently occurs, caused by the air penetrating beneath the cover-glass. This produces changes in many of the corpuscles which may be mistaken for hyaline bodies: the central depression becomes paler and less refractive than the periphery of the corpuscles; later a number of corpuscles contain small glistening points, and still later the corpuscles become crenated.

Giemsa's Stain.—This also gives the Romanowsky staining. The formula is as follows:

Azur II.—eosin,	3	gm.;
Azur II.	0.8	"
Glycerin (Merck, chemically pure),	250	"
Methyl-alcohol (Kahlbaum I.),	250	"

The staining fluid is manufactured by Grübler, and it is best to obtain it already prepared. As Giemsa has modified the formula for another purpose, it is desirable to specify "old" Giemsa stain when ordering.

1. The preparation is dried in the air and fixed in absolute alcohol fifteen minutes, or in methyl-alcohol two or three minutes, after which the alcohol is removed with filter-paper.

2. To 1 c.c. of distilled water in a small graduate add 1

drop of the staining fluid and shake gently. This dilution is to be made immediately before proceeding to the next step.

3. Cover the preparation with freshly diluted staining fluid for ten to fifteen minutes.

4. Wash in a stream of water.

5. Remove excess of water with filter-paper, dry in the air, and mount in balsam.

If specially intense staining is desired, add to the distilled water before mixing it with the stain a little potassium carbonate solution in the proportion of 1 or 2 drops of a 1 per cent. solution to 10 c.c. of water.

Giemsa's Method for Staining Protozoa and Bacteria in Sections.—1. Fix pieces of tissue not more than 2 mm. thick in sublimate alcohol, consisting of 2 parts of a concentrated aqueous solution of corrosive sublimate and 1 part of absolute alcohol. The fixation requires at least forty-eight hours. The fixing fluid is to be renewed after twenty-four hours.

The tissue may remain for as long as three months in the fixing fluid without disadvantage if evaporation is prevented.

2. Dehydrate in graded alcohols and xylol. Embed in paraffin. The sections should not be over 4 microns thick—two microns are better. The tissues must not be handled with metal instruments until after they have been cleared in oil of cedar wood.

3. Treat sections with xylol, followed by graded alcohols and water.

4. Ten minutes in a solution consisting of KI, 2 gms.; distilled water, 100 c.c.; Lugol's solution, 3 c.c.

Instead of this mixture, it is possible to use Lugol's solution only (1 to 3 c.c. of it mixed with 100 c.c. of water or 70 per cent. alcohol), or tincture of iodin diluted with alcohol. The use of the weak alcoholic iodin solution is indicated when a more intense blue staining of the cytoplasm is desired. Treatment with the weaker iodin solutions demands naturally a longer time—twenty to thirty minutes.

5. Treat with 95 per cent. alcohol until the yellow color is removed. After a quick wash with distilled water place

sections for ten minutes in a 0.5 per cent. aqueous solution of sodium hyposulphite, then five minutes in tap-water, and for a short time in distilled water.

6. Stain with freshly diluted Giemsa solution two to twelve hours or longer. The dilution should be 1 drop to 1 c.c. of water; or for a longer period of staining, 1 drop to 2 c.c. of water. After the first half hour the staining mixture is to be poured off and replaced by fresh.

7. Wash in distilled water and dehydrate as follows:

(*a*) Acetone 95 c.c. plus xylol 5 c.c.
(*b*) Acetone 70 c.c. plus xylol 30 c.c.
(*c*) Acetone 70 c.c. plus xylol 30 c.c.
(*d*) Xylol pure.
(*e*) Cedar oil.

8. Mount in cedar oil.

The duration of the treatment with *a*, *b*, and *c* depends upon the degree of differentiation required.

The distilled water used for diluting the staining fluid must be absolutely free from acid. The slightest trace of organic or mineral acids, or even the presence of a considerable amount of carbonic acid, spoils the staining. The distilled water may be tested and corrected for use as follows:

Place 300 c.c. of it in each of 4 flasks. Add 1 per cent. solution of carbonate of sodium (Na_2CO_3), 1 drop to first flask, 2 drops to second flask, and so on. Then take 10 c.c. from each flask in a clean test-tube and add 2 or 3 drops of a fresh solution of hematoxylin in absolute alcohol, which should be pale yellow to nearly colorless. Stand against a white background, and that flask with the right reaction should take on a violet tinge after one to five minutes.

For bringing out certain granules, etc., in special objects a larger amount of alkali in the water is necessary. In this case add to 20 c.c. of the water, shortly before mixing with the staining fluid, an additional drop of alkali solution.

S. B. Wolbach suggests, after considerable experience with the method, the following modifications:

2. Clear in cedar oil instead of xylol.

4. Use the weak solution of iodin in 70 per cent. alcohol.

6. The dilution should be 60 drops of the stain to 100 c.c. of distilled water, 10 c.c. of methyl alcohol, and 2 drops of a 0.5 per cent. solution of sodium carbonate. The stain is replaced twice by fresh mixtures during the first hour.

7. Transfer the sections for differentiation directly from the staining mixture or, after a rapid passage through distilled water, into two changes of the following solution:

Colophonium,	15 grams;
Acetone,	100 c.c.

The slides should be treated individually, and the differentiating fluid should be renewed as soon as the colophonium precipitated by the water fails to dissolve quickly. Differentiation takes place rapidly, fifteen to thirty seconds are usually sufficient. Should a deeper staining with blue be desired the amount of colophonium should be reduced.

8. Pass the sections rapidly through—

Acetone,	70 c.c.
Xylol,	30 "

followed by pure xylol and then by oil of cedar wood. Mount in oil of cedar wood.

Spirochetes of Relapsing Fever.—These spirilla, first discovered by Obermeier in 1873, occasionally are seen clinic-

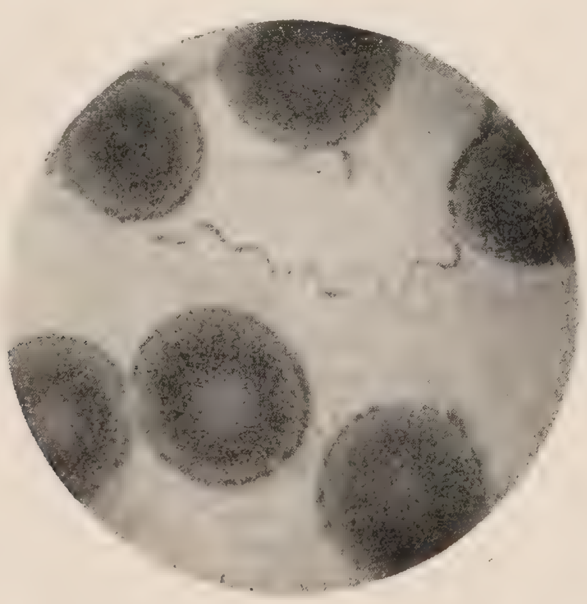


FIG. 133.—Spirochetes of relapsing fever. Smear preparation from blood; stained by Wright's blood-stain. × 1500. (Photo. by L. S. Brown.)

ally in this country. They are present in varying numbers in the circulating blood before and during the febrile paroxysms to which the organism gives rise.

Rabies (Hydrophobia).—The diagnosis of this disease from a pathological standpoint is usually made by the production of experimental rabies in a rabbit by intradural inoculation with material from the nervous system of the animal suspected to have died of it. The poison of the disease is found in the brain, spinal cord, salivary glands, and pancreas. For purposes of inoculation a piece (1 or 2 c.c.) of the medulla or brain, preferably the former, is rubbed up in a sterilized mortar with about 10 c.c. of sterilized distilled water. The resulting fluid is filtered through absorbent cotton, and then through filter-paper, to remove tissue-shreds. Of the clear fluid thus obtained 4 or 5 drops are injected beneath the dura of a rabbit by means of a hypodermic syringe, the skull being trephined with a small trephine about 4 mm. in diameter. The most favorable place for opening the skull is at a point in the median line just posterior to a line drawn through the middle of each eye.

The symptoms of experimental rabies in the rabbit first manifest themselves after two weeks, never earlier, but they may not appear until later, not even until two months have passed. The first symptom is a weakness of the hind legs, followed by paralysis. The paretic condition soon extends to the fore legs, dyspnea appears, and death usually occurs in about three days after the onset of the symptoms. Paralytic symptoms developing before two weeks are not due to infection with rabies, but to some other cause; for instance, infection with the pneumococcus or other bacteria which may be present in the material inoculated.

During the course of the disease the animal never appears stupid, with dull eyes, as in other infections, but remains "conscious," so to speak, until the last.

It is claimed that the diagnosis of rabies may be made also by finding in the nerve-cells of the central nervous system peculiar bodies which are regarded as protozoa, and which are known as "Negri bodies," from the name of their discoverer. These bodies are generally round or oval, but may be irregular, pear-shaped, or triangular in form. They vary in diameter up to 23 μ. They contain small vacuoles,

in some of which are granules of varying size and number; generally there is a central larger structure surrounded by smaller ones. In preparations stained by the eosin-methylene-blue method the bodies generally stain deeply with eosin, with the exception of the granules, some of which stain with the methylene-blue.

Method of Demonstrating Negri Bodies (A. W. Williams and M. M. Lowden).—The bodies may be sought for in smear preparations or in sections. Pieces of gray brain-substance should be taken for examination from the cortex in the region of the fissure of Rolando (in the dog from

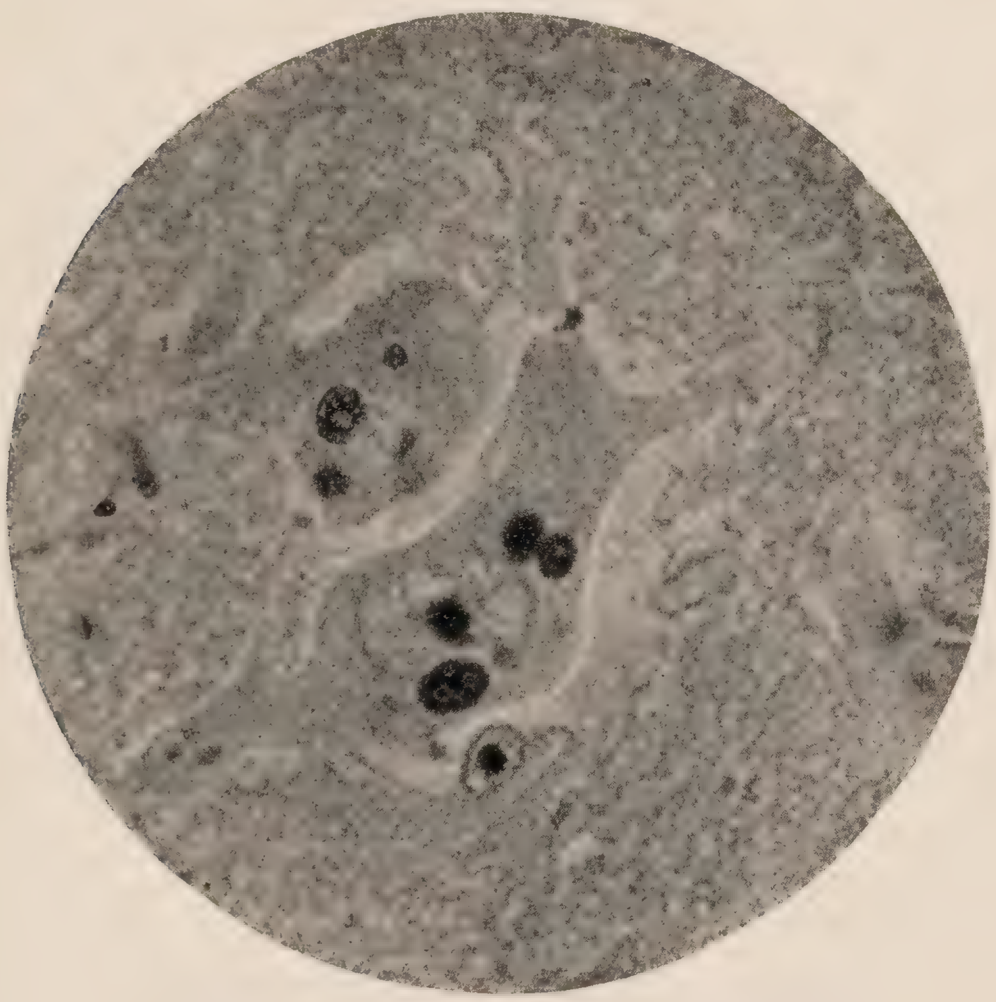


FIG. 134.—Ganglion-cells containing Negri bodies.

around the crucial sulcus), from the hippocampus, and from the cerebellum.

For demonstrating the bodies in sections the tissue should be fixed in Zenker's fluid, imbedded in paraffin, and stained by the eosin-methylene-blue method.

For demonstrating the bodies in smear preparations the following procedure is said to give the best results: A small

bit of the gray substance of brain chosen for examination is cut out with a small, sharp pair of scissors and is placed about 1 inch from one end of a slide. The cut in the brain should be made at right angles to the surface and a thin slice taken, avoiding the white matter as much as possible. A cover-slip is now pressed down upon the piece of tissue until it is spread out in a moderately thin layer, then the cover-slip is moved slowly and evenly over the slide to its other end. The preparation is then dried in the air and fixed in methyl-alcohol for about five minutes. It is then stained by Giemsa's method for malarial parasites (see page 448). It may also be fixed in Zenker's fluid, washed in alcohol, and stained by the eosin-methylene-blue method as a section affixed to the slide.

Entamœbæ.—At least two species of amebæ occur in the intestine, entamœba coli and histolytica. The first is a harmless parasite; the other may give rise to amebic dysentery.

In cases of dysentery suspected of being due to amebæ the stools are best examined as soon as voided, although the amebæ will sometimes remain active in stools even over twenty-four hours old. A warm stage during the examination is an advantage, but not a necessity. A drop of the fluid material, preferably that containing mucus or blood, is placed on a slide and lightly covered with a cover-glass. If the slide is cold and the organisms do not move, warm the slide gently and the movements of the amebæ will often start up. Pus from abscesses due to the amebæ is examined in the same way. A positive diagnosis rests on the presence of the characteristic large, pale cells, consisting of nucleus, granular endosarc, and hyaline ectosarc, and on the movements of the protoplasm, which projects itself more or less actively in the form of pseudopodia.

In hardened preparations the nuclei of the amebæ do not stain with the ordinary nuclear stains, such as alum-hematoxylin and methylene-blue. The following method of staining them has been found to give very satisfactory results and to render the recognition of the organisms easy:

DIFFERENTIAL FEATURES OF ENTAMŒBA COLLI AND ENTAMŒBA HISTOLYTICA.[1]

Name.	Size.	Pseudopodia.	Motility.	Protoplasm.	Nucleus	Cyst formation.	Cultures.	Methods of Reproduction.	Pathogenesis.	Staining.
Entamœba coli, Schaudinn, 1903.	Ten to 30 microns, generally smaller than entamœba histolytica or entamœba tetragena.	Small, blunt, and not clearly differentiated from rest of parasite.	Sluggish.	Ectoplasm not distinct, except when moving, and then only because it is free from granules. Is grayish in color and not very refractive. Endoplasm is gray, finely granular, few non-contractile vacuoles. Is not generally phagocytic for red blood-corpuscles.	Distinct, having a well-defined nuclear membrane and much chromatin. Large karyosome.	Present. Eight young amebæ developed within cyst.	Doubtful.	By simple division; autogenous sexual reproduction in cyst; and by schizogomy with the production of eight daughter amebas. Eight amebas are produced within the cyst.	Is not pathogenic, occurring in a large percentage of healthy individuals.	With Wright's stain, ectoplasm, light blue; endoplasm, dark blue; and nucleus red.
Entamœba histolytica, Schaudinn, 1903.	Ten to 70 microns, generally from 15 to 40 microns.	Blunt or slender and finger-shaped. Very refractive and clearly differentiated from rest of the parasite	Active.	Ectoplasm is very distinct and refractive, in some instances even when motionless. Glassy appearing. Endoplasm is granular, contains numerous non-contractile vacuoles and red blood-corpuscles, when latter are present in feces.	Indistinct. No well-defined nuclear membrane and but little chromatin. Minute karyosome.	Minute spores developed by budding measure 3 to 5 microns. Possess a resistant membrane like a cystic covering. Development of the spores have not been studied.	Doubtful.	By simple division; gemmation; and by the budding of chromidial masses surrounded by protoplasm from the periphery of the mother parasite, forming minute spores.	Is the cause of a form of amebic dysentery.	With Wright's stain, ectoplasm, dark blue; endoplasm, light blue; and nucleus, pale red or pink.

[1] Charles F. Craig, M. D., *The Arch. of Inter. Med.*, vol. vii., No. 3, March 15, 1911.

Differential Stain for the Entamebæ (*Mallory*).—1. Harden in alcohol.

2. Stain sections in a saturated aqueous solution of thionin three to five minutes.

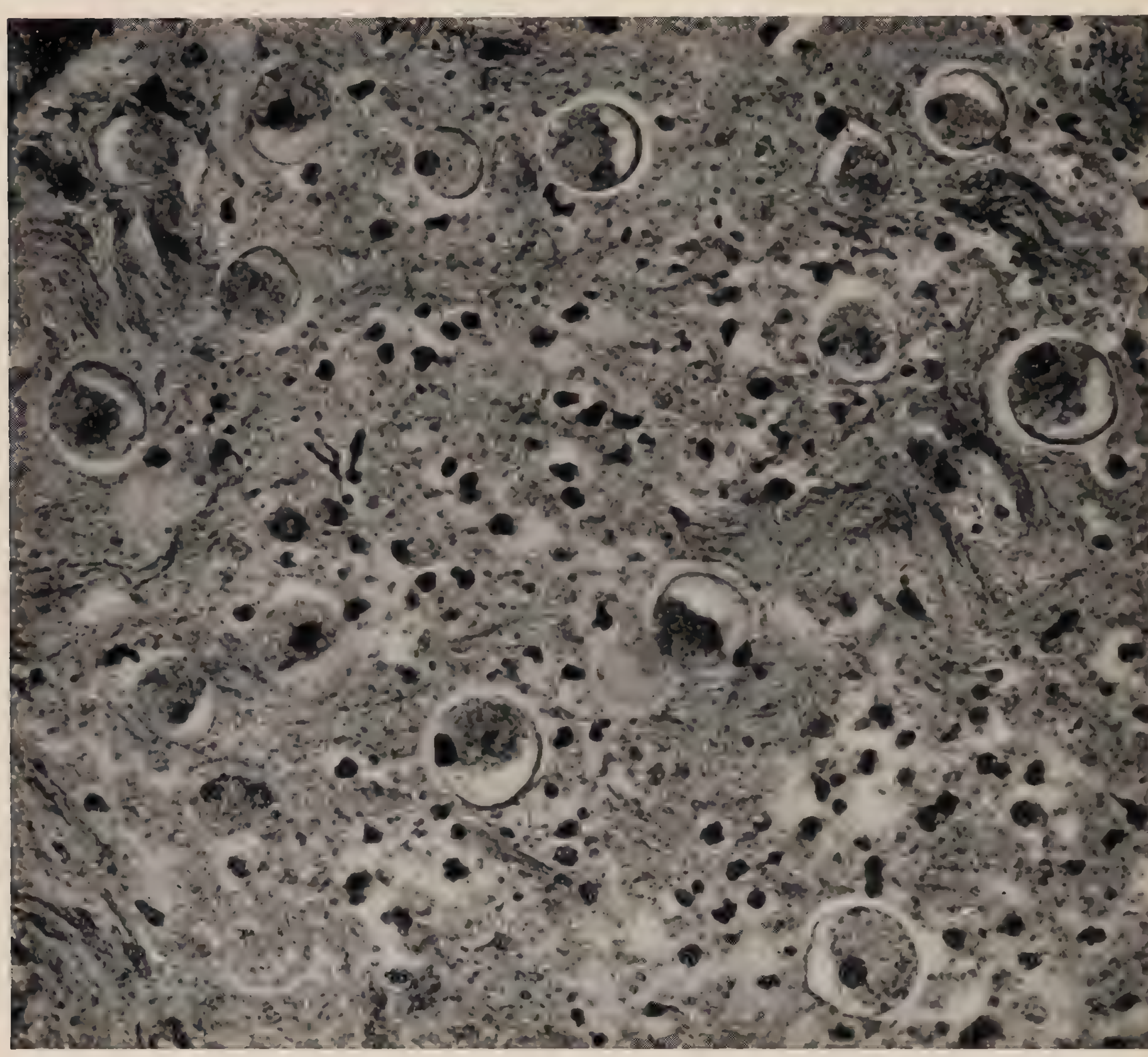


FIG. 135.—Entamœba histolytica in section of ulcer of intestine; × 500 (photo by F. B. Mallory).

3. Differentiate in a 2 per cent. aqueous solution of oxalic acid for one-half to one minute.

4. Wash in water.

5. Dehydrate in absolute alcohol.

6. Clear in xylol.

7. Xylol-balsam.

The nuclei of the amebæ and the granules of the mast-cells are stained brownish red; the nuclei of the mast-cells and of all other cells are stained blue.

Excellent results were obtained by this method with bits of the purulent discharge from a so-called amebic abscess of the liver. After hardening in 95 per cent. alcohol, small fragments the size of a pin-head and less were stained as above directed, and teased apart after they were in the balsam. The reddish nuclei stood out so sharply in the bluish background of fragmented nuclei and granular detritus that they were easily picked out with the high dry power.

The results obtained with feces examined in the same way or after imbedding in celloidin were much less satisfactory, for the reason that various substances in the feces precipitate the thionin in the form of reddish crystals and give rise to deceptive pictures. A similar differential stain can be obtained by Unna's method for staining the granules of mast-cells (see page 336).

Other protozoa, such as the cercomonas and trichomonas, are best examined in fresh preparations.

The Cultivation of Entamœbæ.—The method of W. E. Musgrave and M. T. Clegg is as follows: Make Petri plate cultures on nutrient agar by streaking the surface of the medium with the material containing amebæ. The agar should be 1 per cent. alkaline to phenolphthalein, and should be of the following composition:

Agar,	20;
Sodium chloride,	3;
Beef extract,	3;
Water,	1000.

The material containing amebæ should be prepared by placing it in sterile flasks, adding to it 1 c.c. of alkaline bouillon to each 100 c.c., and setting it aside for twenty-four to forty-eight hours. A loopful of the material from the surface should be taken for the inoculation of each Petri plate. The plates are kept at a temperature not over 37° C. The temperature should be so regulated that the bacteria do not grow so pro-

fusely as to interfere with the growth of the amebæ. After two or three days, if growth of amebæ has occurred, transplant to fresh plates.

Cultures of the amebæ containing only one kind of bacteria may be obtained in the following manner: With a loop, infected with a pure culture of the bacterium with which it is desired to cultivate the amebæ, make several concentric ring-shaped inoculations of different diameters on a sterile agar plate, then inoculate the center of the plate with the mixed bacteria and amebæ culture and incubate. After twenty-four to forty-eight hours the amebæ will have multiplied and wandered out over the surface of the agar to the periphery of the plate, passing on their way through the rings of growth of the pure culture of the bacteria, whereby they tend to lose their mixed bacterial content and take up the bacteria of the pure culture. Amebæ which have passed the outer ring of bacterial growth are to be transplanted to the center of fresh plates inoculated with a pure culture, as above described. This is repeated until a plate culture containing a pure culture of the bacterium is obtained.

Sporozoa.—The *coccidium oviforme* should be examined both fresh in cover-slip preparations and in sections after hardening. The cyst-forms often stain well by the tubercle-bacillus method. For studying all the stages in the development of the organism, Pianese recommends highly his special fixing reagent and stains (see p. 304). Other sporozoa should be studied in the same manner, or by the eosin-methylene-blue stain after fixation in Zenker's fluid. Cover-slip preparations are often useful.

Schistosoma Hæmatobium (Distomum Hæmatobium, Bilharzia).—The male and female parasites occur in the branches of the portal system, especially in the veins of the bladder and rectum, and in the liver. The ova escape from the blood-vessels into the bladder and occasion violent inflammation. The process may extend to the kidneys. The ova also infect the rectum, causing a sort of dysentery, and may involve even the appendix. The ova, with their pointed

spines, are characteristic, and may be found by microscopical examination in the urine and feces. The spines are usually situated at one end, but may occur anywhere in the periphery.

FIG. 136.—Schistosoma hæmatobium. Ovum from fæces showing pointed spine on one side ; × 300 (photo. by L. S. Brown).

FIG. 137.—Schistosoma hæmatobium. Ovum from urine showing terminal spine ; × 375 (photo. by L. S. Brown).

Round-worms.—The embryos of the *filaria sanguinis hominis* or *filaria Bancrofti* (Fig. 138) are examined for in suspected cases by mounting a drop of the fresh blood or

of the chylous or bloody urine on the slide and examining under low power. They are readily detected when present on account of their very active movements. Six species

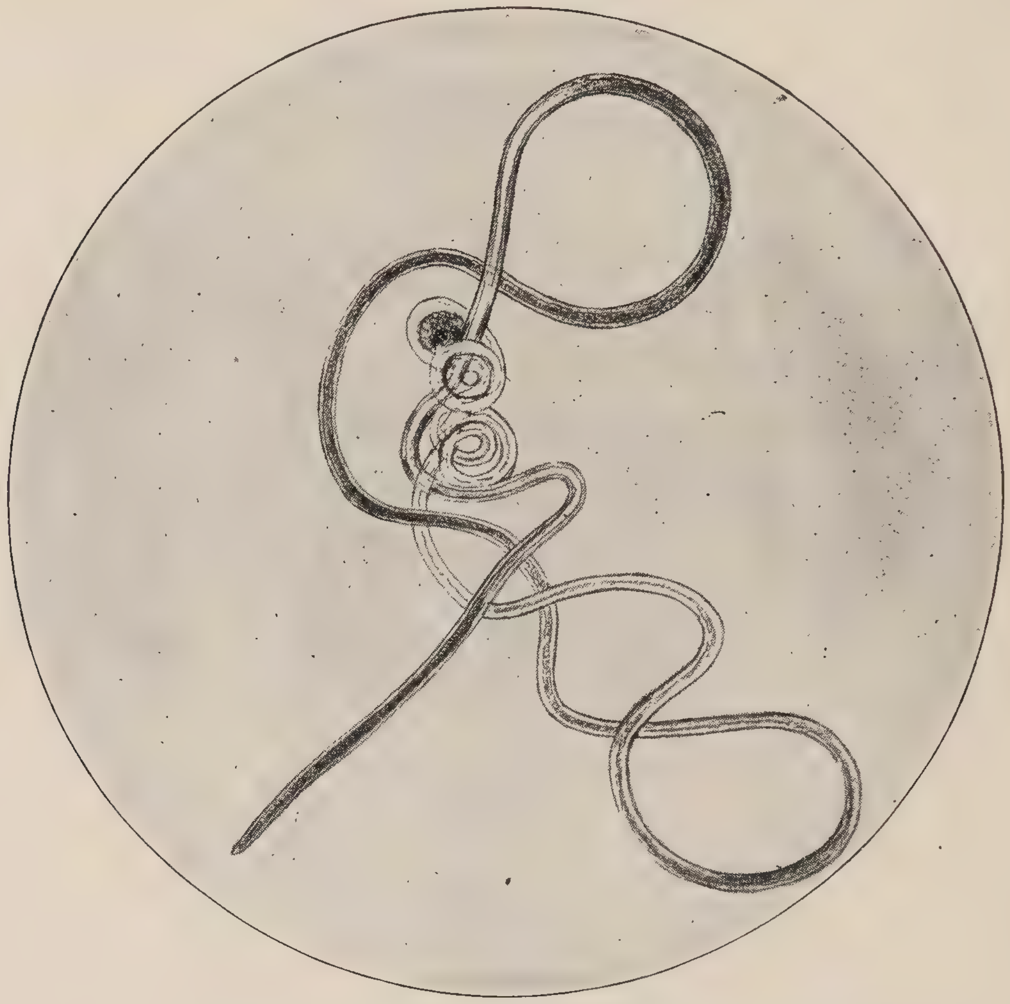


FIG. 138.—Adult male filaria Bancrofti; × 10 (after Lothrop and Pratt).

have been described, but the filaria nocturna is the only one that is known to be pathogenic. The blood should

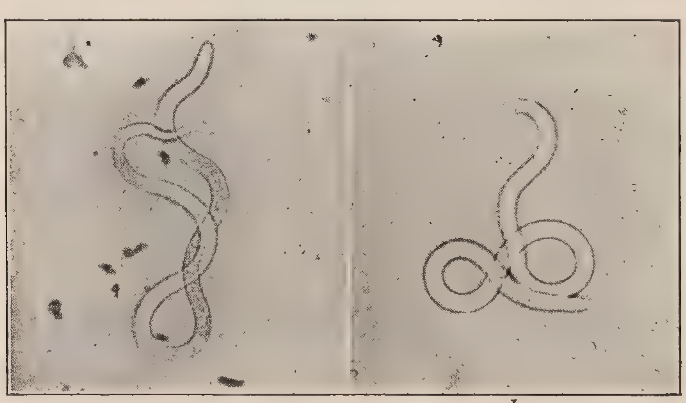


FIG. 139.—Photomicrographs of living filariæ sanguinis hominis; × 250, *a*, from hydrocele fluid; *b*, from blood (after Lothrop and Pratt).

be examined during the resting hours of the patient, as at night for day-workers and during the day for night-workers. Permanent specimens can be made by fixing

ordinary cover-slip preparations of the blood or chylous fluid by heat or by the use of a saturated solution of corrosive sublimate, and staining for a few seconds with Löffler's methylene-blue or with a 2 per cent. aqueous solution of thionin.

In the case of suspected *hookworm disease* the feces are to be examined microscopically for the eggs of the parasite, or

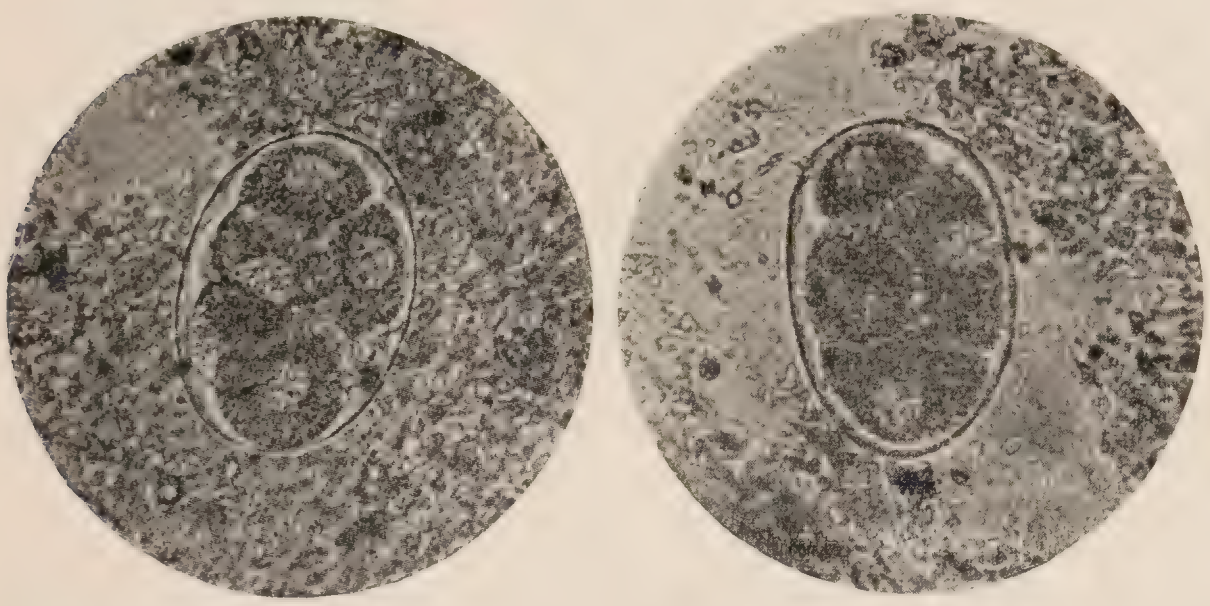


FIG. 140.—Two eggs of the hookworm in feces, each containing several embryonic cells; × 375. (Dr. C. L. Overlander.)

for the adult worms after the administration of an anthelmintic. The finding of the eggs may be facilitated by mixing the feces with a nine-tenths saturated aqueous solution of sodium chloride in a test tube and examining microscopically the superficial portions of the fluid, where any eggs present will accumulate because their specific gravity is less than that of the fluid. This procedure was first recommended by Bass. In fresh feces, eggs containing several embryonic cells are most common. (See Fig. 140.)

Trichinellæ (Figs. 141, 142) are obtained from the fresh muscle by means of teasing. A quick method is to squeeze small bits of tissue between two slides and examine with a low power. Pieces of muscle nearest the insertion of the tendon are chosen from the diaphragm or from the muscles of the jaws. Encapsulated and calcified trichinellæ are cleared up by means of acids.

In hardened tissues the trichinellæ are best studied in longitudinal sections of the muscle-fibers.

The other round-worms which sometimes occur in the

FIG. 141.—Living embryos (Heller).

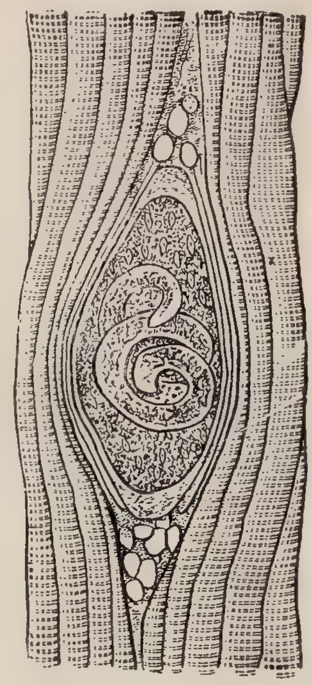


FIG. 142.—Encapsulated trichina (Leuckart).

intestinal tract can be recognized with the naked eye. Their eggs must be looked for with the microscope.

The embryo trichinellæ may be demonstrated in the blood by withdrawing some blood with a syringe from a vein in the

A *B* *C*

FIG. 143.—Tænia solium: *A*, head enlarged; *B*, ripe joint, × 6; *C*, egg of tænia solium (Heller).

arm, washing it with 3 per cent. acetic acid, centrifugalizing, and examining the sediment (W. H. Herrick and T. C. Janeway).

Tape-worms.—It is not always easy to recognize the kind of tape-worm by a single segment passed with the feces,

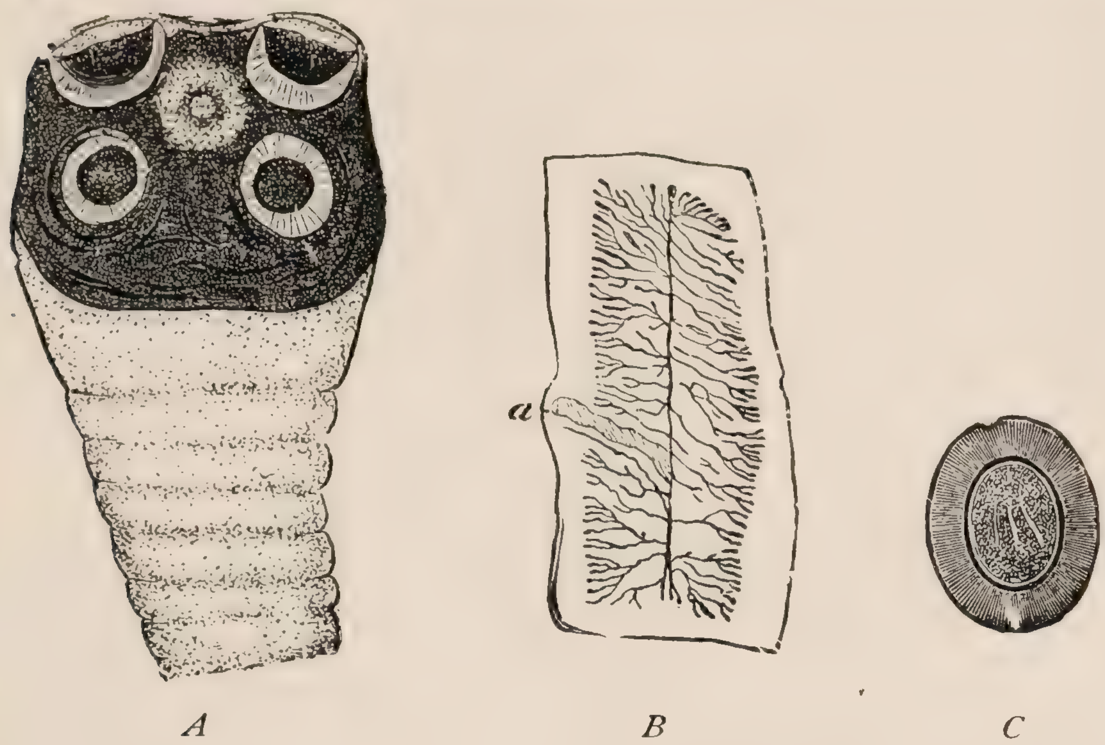


FIG. 144.—Tænia mediocanellata: *A*, head darkly pigmented; *B*, ripe joint, × 6; *C*, egg of tænia mediocanellata.

because the uterus, which furnishes the most characteristic points of difference, is not developed in the young segments and is atrophied in the old ones. When the whole worm is obtained the problem is much simpler. The uterus is best

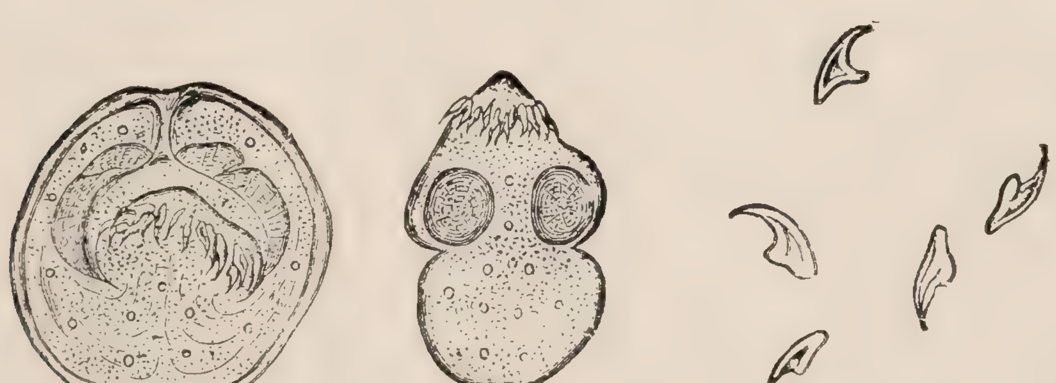


FIG. 145.—Echinococcus: scolices, hooks (Heller).

made out by squeezing a segment between two slides and holding it up to the light. The heads are examined under the microscope in water, salt solution, or glycerin.

Tænia Solium (Fig. 143).—Head has four suckers and a circle of hooklets; uterus is noticeably but little branched. The genital tract opens laterally. The eggs develop into the

cysticerci cellulosæ, which are not infrequently found in man. The scolex is obtained for examination by tearing open the

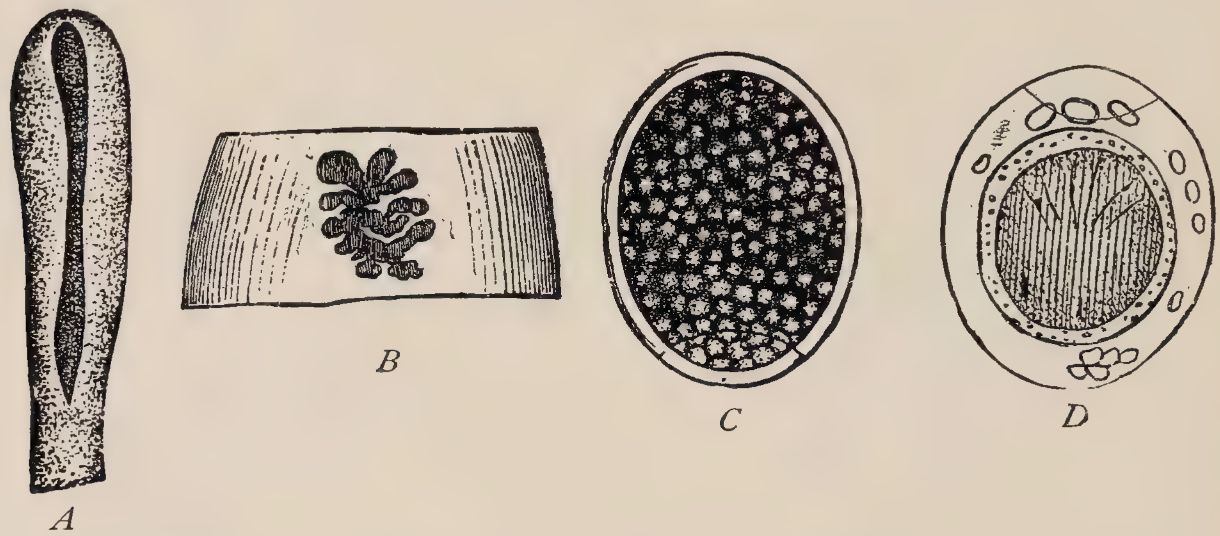


FIG. 146.—Dibothriocephalus latus: *A*, head; *B*, ripe joint, × 6; *C*, egg of dibothriocephalus latus (Heller); *D*, egg with developed embryo (Leuckart).

cyst and examining the inner wall. The suckers and hooklets are best studied after mounting fresh and pressing under a cover-glass.

FIG. 147.—Segments of tænia saginata (after Stein).

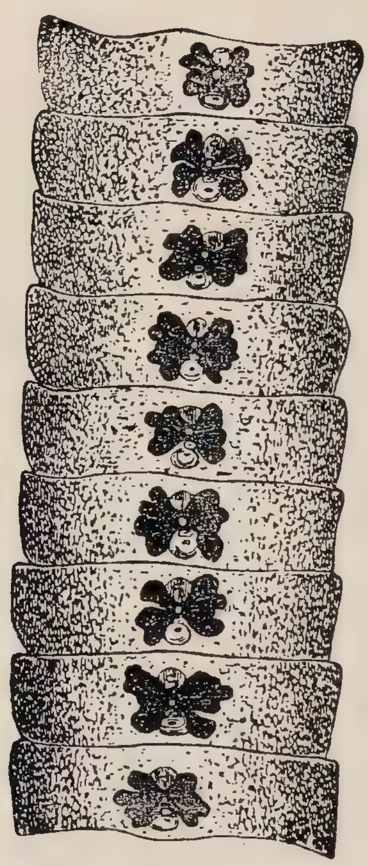


FIG. 148.—Segments of bothriocephalus latus (after Stein).

FIG. 149.—Segments of tænia solium (after Stein).

Tænia Mediocanellata s. Saginata (Fig. 144).—Head has four strong suckers, but no hooklets; uterus is very

much branched, segments show marked muscular development. The genital tract opens laterally. The eggs develop into *cysticerci*, which do not occur in man.

Tænia Echinococcus (Fig. 145) occurs in dogs. The echinococcus cysts which occur in man are recognized by the very characteristic laminated structure of the cyst-wall. The heads of the scolices have four suckers and a double circle of hooklets.

a *b* *c* *d* *e*

FIG. 150.—Comparative size of eggs of intestinal parasites: *a*, tænia solium; *b*, tænia mediocanellata; *c*, ascaris lumbricoides; *d*, trichocephalus dispar; *e*, oxyuris vermicularis (after Strümpell).

Dibothriocephalus latus (Fig. 146).—The opening of the genital tract lies in the median line. The head is flattened, and has two small suckers situated at the sides.

CLINICAL PATHOLOGY.

The material received at the pathological laboratory from clinical cases consists largely of new growths and lesions produced by infectious, chemical, or traumatic agents—to a less extent of fluids from various natural or artificial cavities or secretions and eliminations from the respiratory, genito-urinary, and gastro-intestinal tracts. In the following pages a brief synopsis is given of what may be expected in the various fluids and eliminations, and the more important chemical tests required for the recognition of certain substances are added.

Gross specimens removed at operations should be treated as parts of autopsies, and dissected or otherwise examined bacteriologically and histologically in an equally careful

manner. For the finer study of cancers and other new-growths fixation in Zenker's fluid and staining by the following methods is particularly recommended: eosin-methylene blue, phosphotungstic acid hematoxylin, and anilin blue stain for collagen fibrils. In addition the methods of Pianese may be employed (see page 304).

Examination of Tissues from Clinical Cases for Diagnosis.—Tumors of any size or large pieces of tissue present no difficulties. There is plenty of material to examine fresh or after fixing in a variety of ways. Frozen sections of the fresh tissue are often sufficient. Sometimes it is better to harden for an hour or more in formaldehyde and then to make frozen sections.[1] Often it is wisest to harden in strong alcohol and then to make razor sections or to embed in celloidin. The whole process of hardening, embedding, and sectioning can easily be carried through in twenty-four hours with small pieces of tissue. Embedding in paraffin is sometimes preferred. In cutting sections of small pieces it is important to mount them if possible, so that the cut sections will show proper relations—*i. e.*, vertical sections through the skin, uterine mucous membrane, etc.—otherwise confusing pictures will often be presented. It is important to know, in regard to pieces of tissue sent for diagnosis, from what part of the body they come.

A hematoxylin-and-eosin stain will be found the most generally useful for hardened sections.

Uterine Scrapings.—Small pieces may be examined fresh in frozen sections or after hardening for one or more hours in formaldehyde. Better results are obtained by hardening in alcohol and imbedding in celloidin or paraffin. Where the fragments are small, it is advisable to mass them together on a small piece of filter-paper and to harden in formaldehyde or in Zenker's solution. The mass can then be embedded in celloidin and cut as one piece of tissue, or they can be carried through by the gelatin-formaldehyde embedding method (see p. 290), and cut on the freezing microtome. A hematoxylin-and-eosin stain is the best, because the eosin

[1] For frozen section methods, see p. 271.

brings out the smooth muscle-fibers prominently, so that any invasion of the muscular coat by a malignant growth is more readily made out—a valuable help in the diagnosis of malignant adenoma.

Examination of Fluids obtained by Puncture.—The **transudations** obtained largely from the serous cavities are non-inflammatory in origin. They are usually of a transparent, pale-yellow color with slightly greenish tint, alkaline in reaction, and deposit on standing a slight flocculent coagulum.

The *specific gravity*, to be taken at room-temperature, varies according to the origin of the fluid. According to Reuss, it is below 1015 in hydrothorax; below 1012 in ascites; below 1010 in anasarca.

The amount of *albumin* in hydrothorax is always under 2.5 per cent., and in ascites between 1.5 and 2 per cent. Microscopically, a few leucocytes, usually fatty degenerated and rarely desquamated endothelial cells, are found.

The **exudations** are of inflammatory origin, and are also generally obtained from the serous cavities. From their various microscopic appearances they are divided into serous (fibrino-serous), hemorrhagic, purulent, and gangrenous. The specific gravity of all is over 1018; the reaction is always alkaline. On standing they deposit a varying amount of sediment. Examinations for organisms should always be made. Occasionally a peculiar opalescent layer, due to cholesterin crystals, forms on the surface of fluids which come from old cases of pleurisy.

Serous Exudations.—The fluid, which immediately after removal is slightly cloudy and yellowish in color, deposits more or less quickly a flocculent or dense coagulum. Microscopically, the coagulum shows a dense meshwork of fibrin and numerous polynuclear leucocytes.

Hemorrhagic Exudations.—The sero-fibrinous exudation is colored a lighter or darker red according to the amount of blood present. Microscopically, the same elements are found as in the serous exudations, plus a marked increase

of red blood-globules, which are usually well preserved, but in old exudations may be more or less decolorized.

Aside from injuries, hemorrhagic exudations are most common in connection with tuberculosis and new growths, so that their microscopic examination is of much diagnostic and prognostic value.

For the examination for tubercle bacilli see page 184. In this form of exudation it is rarely possible to demonstrate them. On the other hand, it is not infrequently possible to make the diagnosis of a malignant growth, especially of cancer, from the examination of the sediment. No cell is significant of cancer or other neoplasm, but the occurrence of numerous cells which vary greatly in form is suspicious. The cells from new growths are often unusually large, up to 120μ, frequently contain one or more vacuoles, and usually lie in clumps. Large drops of fat are also considered suspicious.

A positive diagnosis can only be made by obtaining bits of tissue which show the structure of the new growth, such as the atypical alveolar arrangement of the cells in cancer.

Purulent exudations appear more or less thick and yellow, and deposit a corresponding layer of pus. Microscopically, they present no peculiarities other than the organisms to which they are due. Among the etiological factors actinomyces must always be thought of in puzzling cases.

Putrid exudations occur in the pleural and peritoneal cavities in consequence of gangrenous masses breaking into them and from stomach or intestinal ulcerations, from new growths, occasionally from no clear cause. The fluid resulting from the perforation of a gastric ulcer may show yeast-cells and sarcinæ, and give an acid reaction.

Examination of Serous Fluids.[1]—In 1900 Widal and Ravaut published a method for the examination of serous fluids and gave it the name of *cytodiagnosis.* Recently Jousset has described a method for the detection of the

[1] This section has been written by Dr. Percy Musgrave, who has thoroughly tested these methods in the Clinico-Pathological Laboratory of the Massachusetts General Hospital.

bacillus tuberculosis, under the name of *inoscopy*. These two methods have been found of much importance in the examination of serous effusions as means of determining their etiology.

Cytodiagnosis consists in the examination of the cellular elements with reference to the variety of cell which predomi-

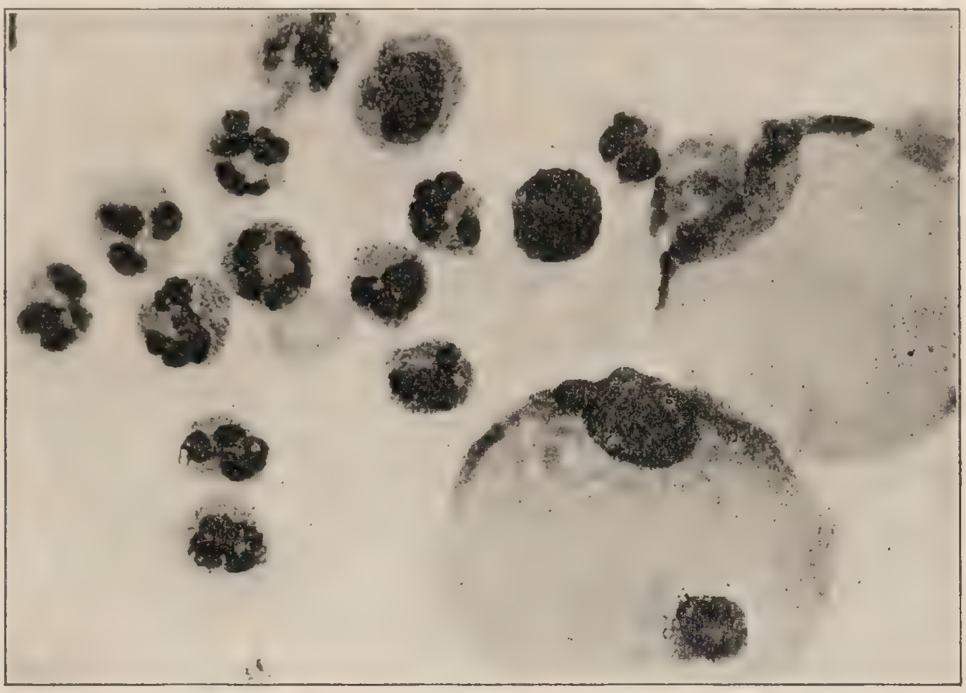


FIG. 151.—Cytodiagnosis. Polynuclear leucocytes and swollen endothelial cells in a smear preparation from the centrifugalized sediment of the fluid from an acute infectious non-tubercular pleuritis (Percy Musgrave; photo by L. S. Brown).

nates in the sediment. The originators of this method have given us the following formulæ:

1. Predominance of polynuclear leucocytes means an acute infectious process.

2. Predominance of lymphocytes means tuberculosis.

3. Few cellular elements with a large proportion of endothelial cells, occurring especially in sheets or plaques, means a transudate or mechanical effusion.

These writers have given us no special formula for cancer, but there is reason to believe that cancerous fluids show a relatively large number of endothelial cells mixed with a larger percentage of lymphocytes than is found in the mechanical effusions, and also that cancerous fluids have a large amount of albumin and a high specific gravity. Further research, however, on this point is necessary.

The age of the effusion in the acute infectious variety has some modifying effects, for which the reader is referred to the original articles.

In the tubercular variety, although there is usually a high percentage of lymphocytes in the first ten days, the polynuclear leucocytes may predominate, but after the second week the formula remains fairly constant.

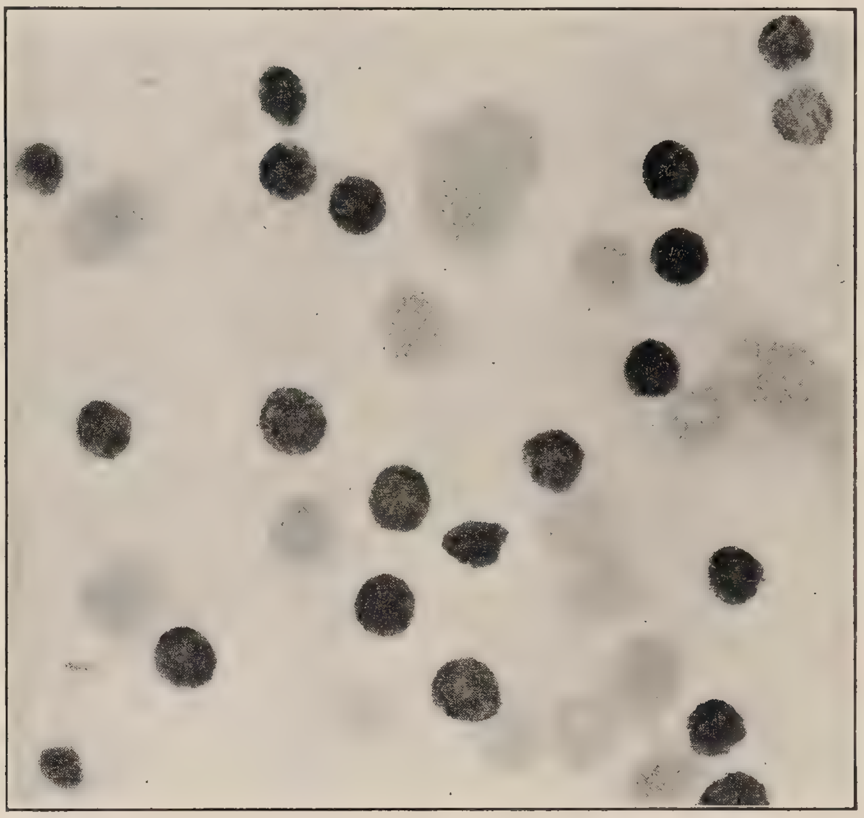


FIG. 152.–Cytodiagnosis. Lymphoid cells in a smear preparation from the centrifugalized sediment of pleural fluid; case of tubercular pleuritis (Percy Musgrave; photo by L. S. Brown).

The so-called secondary tuberculous pleurisy (caused by direct extension from a tuberculous focus in the lung) often shows a large number or even a predominance of polynuclear leucocytes, owing to a secondary infection with pyogenic bacteria.

Old mechanical effusions are occasionally encountered where little endothelium is seen, and the cells found are almost exclusively lymphocytes.

Method.—The fluid should be drawn with the usual aseptic precautions into sterilized flasks or tubes. If it is clotted, it should be shaken until the clot is thoroughly contracted, and the clot, or all clots of large size, should be removed.

Place the fluid in centrifuge tubes and centrifugalize for five minutes at least.

Decant the supernatant fluid gently at first, and when a small amount only remains, invert the tube for about two seconds. A few drops only will be left.

With a small platinum loop stir the sediment thoroughly, rubbing the sides of the glass to remove adherent portions. When the sediment is thoroughly mixed with the few drops

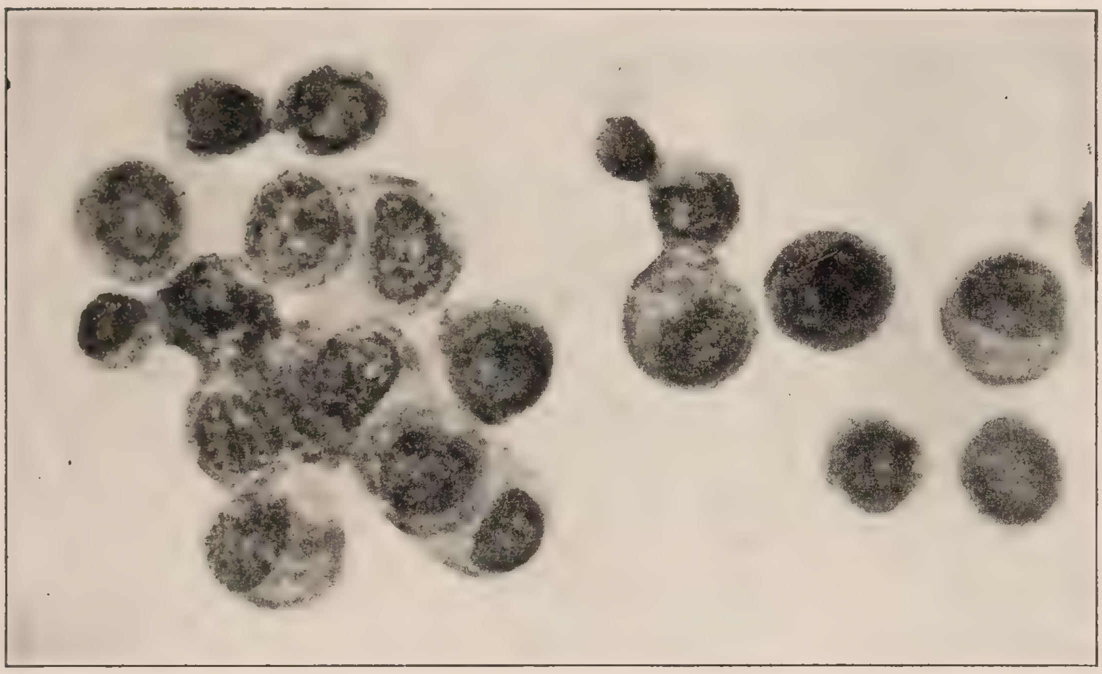


FIG. 153.—Cytodiagnosis. Endothelial cells in a smear preparation from the centrifugalized sediment of a transudate or mechanical effusion (Percy Musgrave; photo by L. S. Brown).

of fluid remaining after decantation, remove a drop of the mixture with the platinum loop and make a cover-slip smear. Allow this to dry spontaneously or by very gently heating. Heating at the boiling-point will spoil the preparation.

Cover the preparation with a staining fluid made as follows:

Wright's blood-stain,	3 parts;
Pure methyl-alcohol,	1 part.

Allow to remain on the preparation twenty to forty-five seconds, then dilute it with 8 to 10 drops of water and allow this mixture to stand one to two minutes.

Wash very gently, preferably by flooding the slide with a dropper. Do this four or five times, allowing the water to remain on the slide a few seconds each time. Vigorous or

forcible washing will destroy the film and spoil the preparation.

Dry the preparation by holding it between the thumb and forefinger and waving it through the Bunsen or alcohol flame. Do not attempt to blot the preparation or heat it above the temperature which the fingers will bear.

Mount in xylol-balsam and examine with an oil-immersion lens.

Inoscopy is practised as follows:

1. The fluid should be drawn with asceptic precautions into sterilized flasks (Erlenmeyer flasks preferably), and at least 100 c.c. should be taken, although results may sometimes be obtained with much smaller amounts. Allow the fluid thus taken to clot.

2. Shake the fluid gently to contract the clot as much as possible, and then wash it, on a piece of sterile linen or fine gauze wrapped over the end of a funnel, until all the serum is washed away.

3. Remove the clot or clots with a sterile spatula and place in a small flask with sufficient of the following fluid to digest it:

Pepsin,	2 gm.;
Pure glycerin, Strong HCl, each,	10 c.c.;
Sodium fluorid,	3 gm.;
Distilled water,	1000 c.c.

The amount of this fluid necessary will vary, of course, with the size of the clot to be digested, but 20 or 30 c.c is sufficient in most cases. A freshly prepared pepsin—HCl solution—apparently serves as well as the above fluid.

4. Place the above preparation in the incubator or oven until the clot is digested. A temperature of 37° C. for two or three hours will suffice, but the time is shortened if kept at a temperature of 50° C.

5. When the clot has disappeared, pour the mixture into centrifuge tubes and centrifugalize for five to ten minutes. Decant the supernatant fluid as described under cytodiagnosis (see p. 470).

6. Make a cover-slip preparation and stain it for tubercle bacilli. Care, however, should be taken not to decolorize too long—one-half to three-quarters of a minute with Gabbet's solution is sufficient. Dry and mount.

The majority of the bacilli found by this method are shorter and broader, as a rule, than the tubercle bacilli ordinarily seen in sputum, and some are paler red, but all the forms occur. These bacilli may occur singly or in groups. The greater part of the sediment consists of undigested nuclei and a small amount of detritus.

Animal inoculation gives the most satisfactory results if practised as follows:

Take at least two centrifuge tubefuls of the fluid and centrifugalize for ten minutes. Decant the supernatant fluid and add about 10 m. of the original fluid to each tube. Stir up the sediments in the two tubes until thoroughly suspended; then mix them and inoculate a guinea-pig subcutaneously. Not over 30 m. of fluid should be used, since this is sufficient, and in most cases does not cause toxemia in the animal.

Cultures from purely serous fluids are, in the vast majority of cases, sterile. In a few cases the writer has found the pneumococcus and streptococcus in pure culture.

Lumbar Puncture.—The diagnostic value of lumbar puncture has been sufficiently demonstrated. Not only is it possible to diagnosticate inflammation of the meninges, but the character and cause of the inflammation may usually be demonstrated if the examination of the fluid is properly performed. In a number of cases of general infection in which there was no inflammation of the meninges a diagnosis has been made by means of cultures taken from the cerebro-spinal fluid. Finally, a number of cases of hemorrhage into the brain and spinal canal have been diagnosticated by lumbar puncture.

The operation and the subsequent examination of the fluid should be as carefully performed as any other bacteriological investigation in order to obtain accurate results. The back of the patient and the operator's hands should be made sterile. The needle should be boiled for ten minutes. The

patient should lie on the right side, with the knees drawn up, and with the uppermost shoulder so depressed as to present the spinal column to the operator. This position permits the operator to thrust the needle directly forward rather than from the side. An antitoxin needle 4 cm. in length, with a diameter of 1 mm., is well adapted for infants and young children. A longer needle is necessary for adults and children over ten years of age.

Aspiration of the fluid is not necessary, but some operators prefer to attach a hypodermic syringe to the needle to afford a better grasp for the hand. In this case the syringe would have to be detached to allow the fluid to flow. The additional manipulation, and possibly the defective sterilization of the syringe, might impair the subsequent bacteriological examination.

The puncture is generally made between the third and the fourth lumbar vertebræ; sometimes between the second and third. The thumb of the left hand is pressed between the spinous processes, and the point of the needle is entered about 1 cm. to the right of the median line and on a level with the thumb-nail, and directed slightly upward and inward toward the median line. Care must be exercised to prevent the point of the needle from passing to the left of the median line and striking on the bone. At a depth of 3 or 4 cm. in children and 7 or 8 cm. in adults the needle enters the subarachnoid space, and the fluid flows usually by drops. If the point of the needle meets with a bony obstruction, it is advisable to withdraw the needle somewhat, and to thrust again, directing the point of the needle toward the median line, rather than to make lateral movements, with the danger of breaking the needle or causing a hemorrhage. The smallest quantity of blood obscures the macroscopic appearance of the fluid by rendering it cloudy. The fluid is allowed to drop into an absolutely clean test-tube which previously has been sterilized by dry heat to 150° C. and stoppered with cotton. The fluid should be allowed to drop into the tube without running down the sides. From 5 to 15 c.c. of fluid is a sufficient quantity for examination.

In meningitis there is always an exudation of cells which

makes the fluid more or less cloudy. The degree of cloudiness is to some extent proportionate to the amount and character of the exudation. In tubercular meningitis the amount of cellular exudation is sometimes so slight that the fluid appears clear unless examined carefully.

Cultures on blood-serum and cover-glass preparations should be made from the fluid. In most cases this is best done from the sediment thrown down by the centrifuge. It is of great importance that the tube of the centrifuge should be clean and sterile. If tubercular meningitis is suspected, a guinea-pig may be inoculated with the sediment.

The cover-glass preparations, after drying in the air, are best stained with Wright's blood-stain (see page 382). This reveals the characters of the cells very clearly and stains any bacteria that may be present, with the probable exception of the tubercle bacillus.

A predominance of polynuclear leucocytes in the sediment means non-tubercular meningitis. The infecting bacterium should be sought for and its identity determined. It will generally be the diplococcus intracellularis or the pneumococcus. A predominance of large and small lymphocytes in the sediment indicates the existence of tubercular meningitis, and cover-glass preparations should be stained for tubercle bacilli. It may be necessary to examine twenty or more preparations before finding the bacilli.

The number of cells in the spinal fluid may be estimated by the same method as for white blood-corpuscles.

The albumin in the fluid should be quantitated. Normally, the cerebro-spinal fluid contains from $\frac{1}{40}$ to $\frac{1}{100}$ of 1 per cent. or less. In meningitis the amount is increased from $\frac{1}{30}$ to $\frac{1}{10}$ of 1 per cent., or often more. Percentages of albumin down to $\frac{1}{100}$ of 1 per cent. can be estimated by the ferrocyanide-of-potassium-and-acetic-acid test and a centrifugal machine. Take $3\frac{1}{2}$ c.c. of a 20 per cent. solution of ferrocyanide of potassium, 1.5 c.c. of acetic acid, and 10 c.c. of the fluid in which the albumin is to be quantitated. The mixture of reagents and fluid is poured into a conically shaped graduated glass vessel designed for the purpose and centrifugalized. Sugar is rarely present and has no diagnostic value.

Noguchi Reaction.—To 0.2 c.c. of spinal fluid add 0.5 c.c. of 10 per cent. solution of butyric acid in normal salt solution and boil. Then add 0.1 c.c. of a 4 per cent. aqueous solution of sodium hydrate and boil again. A flocculent precipitate appears in from twenty minutes to two hours, when the reaction is positive.

Alzheimer's Method for the Cytological Examination of the Cerebro-spinal Fluid.—This method is especially useful in the diagnosis of general paralysis. The description of it is taken from a paper by H. A. Cotton and J. B. Ayer. The method is as follows:

1. Lumbar puncture in the usual manner.
2. 96 per cent. alcohol, in proportion to twice the amount of cerebro-spinal fluid, is added drop by drop and well mixed.
3. Centrifuge the mixture for one hour at high speed in a glass tube with conical end. (An ordinary electric urinary centrifuge apparatus can be employed, the tube to be well stoppered to prevent evaporation.)
4. The supernatant fluid is poured off, leaving a small coagulum in the bottom of the tube.
5. Add absolute alcohol—alcohol and ether—ether, each separately for one hour, to dehydrate and harden coagulum.
6. The coagulum can now be gently loosened from the bottom of the tube by a long needle. The tube is then inverted, and the coagulum allowed to fall into the hand by a quick tap on the end of the tube. Care must be taken not to squeeze or handle the coagulum. The hand is placed over a small homeopathic vial, containing thin celloidin, and the coagulum allowed to drop into the celloidin, where it remains over night (twelve hours usually).
7. Coagulum placed in thick celloidin which is allowed to evaporate slowly. It is then mounted on blocks and sections cut 14μ in thickness.
8. The sections are stained and mounted according to the following procedure:

   (*a*) Remove celloidin by absolute alcohol and ether.

   (*b*) 80 per cent. alcohol.

   (*c*) Water.

(*d*) Sections are carried on glass or platinum needle into a dish of Pappenheim's pyronin-methyl green stain (see p. 95) and kept in a water-bath at 40° C. five to seven minutes.

(*e*) Quickly cool dish in running water.

(*f*) Wash off superfluous stain in plain water.

(*g*) Absolute alcohol to differentiate—until no more stain comes away from section.

(*h*) Clear in Bergamot oil.

(*i*) Mount in balsam.

The cells are caught in the coagulum and are nearly evenly distributed throughout it. Cross-sections are prepared and examined from at least 6 levels in the coagulum. The number of cells in 100 fields of a half-inch or similar objective is taken as the unit for comparison. A high cell count—that is, over 100 cells to 100 fields, the presence of plasma-cells and perhaps phagocytes, in a case of suspected general paralysis—is the strongest evidence in favor of this diagnosis.

Ovarian and Parovarian Cysts.—The simple cysts of the ovary due to distention of Graafian follicles or to cystic change of corpora lutea, and the parovarian cysts contain a thin, clear, serous fluid of low specific gravity.

The contents of the multilocular and papillary adeno-cystomata of the ovary are usually tenacious and mucous, of very varying specific gravity, from 1005–1050, but usually between 1020 and 1024. The fluid generally contains much albumin and is rich in metalbumin, which is precipitated by alcohol, but not by acetic acid, nitric acid, or boiling, so that it can readily be distinguished from mucin. Before making the test the albumin must be removed.

The cyst-contents are usually yellowish, but sometimes may be dark-red or chocolate-colored. Microscopically, red and white blood-globules, occasionally blood-pigment and cholesterin crystals, often fat-granules and large vacuolated cells, are found in the cyst fluid. Bizzozero considers cylindrical epithelial cells, ciliated and beaker cells, and colloid concretions especially important from a diagnostic point of view.

Pancreatic Cyst or Fistula.—The fluid obtained from

a permanent fistula or large cyst of the pancreas contains much less solids than the normal pancreatic juice, and the trypsin ferment may be present in very small amount or possibly be entirely wanting. The fluid is colorless, alkaline in reaction, and has a specific gravity of about 1011. It is characterized by three distinct properties on which its recognition depends—namely:

1. It splits up fat into fatty acids and glycerin. Mix together equal parts of neutral olive oil and the alkaline fluid. Test with litmus-paper. Place the mixture in the incubator at 37° C., and test from time to time. If the fluid is pancreatic, an acid reaction will be obtained in twelve to eighteen minutes.

2. It transforms starch into sugar. Place in the incubator equal parts of a 1 per cent. aqueous solution of starch and of the fluid to be tested. In ten to twenty minutes test for sugar with Fehling's solution.

3. It digests fibrin in an alkaline solution (trypsin ferment). Place some fibrin in the alkaline fluid and set it in the incubator. In one-half to one hour examine for peptones by the *biuret test.* Add caustic potash or soda and a few drops of a dilute solution of sulphate of copper. If peptones are present, a beautiful reddish-violet color will be produced.

Dropsy of the Gall-bladder.—Puncture is generally not advisable. The fluid is usually colorless and mucoid or serous in character. All trace of biliary constituents may have disappeared. According to Lenhartz, numerous colon bacilli are usually present.

Hydronephrosis and Renal Cysts.—The fluid is almost always clear as water, rarely reddish or yellow. Specific gravity always under 1020 (usually between 1010 and 1015). Urea and uric acid are generally present, but may be absent. (Small amounts of urea are sometimes present in ovarian cysts.) Albumin is slight in amount. Microscopically, almost nothing is found.

Echinococcus Cysts.—The fluid is perfectly clear, free from albumin, and contains a little succinic acid and much

chlorid of sodium. The specific gravity varies between 1008 and 1013.

Microscopically, often no traces of morphological elements can be found. Occasionally, however, hemosiderin granules or cholesterin crystals occur, or the characteristic structures from which a positive diagnosis can be made—namely, scolices, hooklets, or pieces of cyst-membrane.

A positive diagnosis from a chemical examination depends on showing—

1. The absence of albumin.

2. The presence of chlorid of sodium.

Evaporate a drop of the fluid slowly on a slide, so as to get the characteristic crystals of chlorid of sodium.

3. The presence of succinic acid.

Acidify a little of the fluid with hydrochloric acid and evaporate to dryness. Extract the residue with ether. The crystallized material left on the evaporation of the ether, if dissolved in water, will give a rust-colored, gelatinous precipitate with sesquichlorid of iron if succinic acid be present.

Examination of the Sputum.—The secretion raised from the air-passages by coughing is almost invariably contaminated with the secretion of the naso-pharynx and with particles of food from the mouth. In examinations of sputum these contaminations must always be borne in mind. The amount raised varies from a few c.c. to one or even several liters in twenty-four hours.

The macroscopic appearances of the sputum depend on the varying proportions of mucus, pus, blood, and serum present. The tenacity is mainly due to the mucus. The reaction is usually alkaline.

The general color, consistency, and separation into layers is best seen after the sputum has stood for some time in a tall glass. For more careful macroscopic examination small portions of the sputum are transferred to flat glass dishes, where they are spread out thinly by needles and examined over black or white paper. Porcelain plates painted black or black paper itself can be used. The latter method is convenient, because the sputum can be burned up with the paper.

The constituents of the sputum which may be recognized macroscopically are few in number, and not so important as those which may be found microscopically.

Macroscopic Examination.—1. *Caseous Masses.*—In the sputum from tubercular cases small, opaque, yellowish-white masses from the size of a pin-head to that of a small pea can occasionally be found, which spread out beneath a cover-glass like a bit of cheese. They are small caseous masses which are valuable for microscopic examination because they usually contain tubercle bacilli and elastic fibers.

2. *Fibrinous casts of the bronchioles* can usually be found in the sputum from the third to the seventh day in cases of acute lobar pneumonia. They appear as yellowish-white or reddish-yellow threads, 2 to 3 mm. thick and ½ to several cm. long, and are often branched. The large ones are often rolled into balls, and show best after being shaken in water. Casts of the bronchi are found in cases of fibrinous bronchitis.

3. *Curschmann's spirals* (Fig. 154) of twisted threads of mucus enclosing epithelial cells and leucocytes occur rarely, except in bronchial asthma. They appear macroscopically as grayish-white or whitish-yellow masses or threads, ½ to 1½

FIG. 154.—Curschmann's spirals; *a*, central fiber.

mm. thick and ½ to 8 cm. long, and often show a visible spiral arrangement.

4. *Dittrich's Plugs.*—These are whitish-yellow masses from the size of a pin-head to that of a bean, which are formed in cases of putrid bronchitis and of gangrene of the lung.

They have a very fetid odor, a cheesy consistency, and are rather easily compressed. Besides organisms they contain numerous fat-crystals.

5. *Shreds of tissue* are found almost solely in gangrene of the lung, and are best recognized with the microscope.

6. *Concretions*, portions of cysticercus membrane, etc., are rare in the secretion from the lungs.

Microscopic Examination.—Microscopically, the sputum may show various kinds of cells, fragments of tissue, including elastic fibers, vegetable and animal parasites, and crystals.

They will be taken up in order:

1. *Red Blood-globules.*—In fresh hemorrhages they appear normal, often in rouleaux. In old sputa many have lost their color.

2. *White blood-globules* are almost invariably polynuclear, and the majority of them contain neutrophilic granules. In asthma, however, numerous eosinophilic and rather numerous basophilic leucocytes are regularly found. The leucocytes often contain pigment- or fat-granules.

3. *Epithelial Cells.*—Pavement, cylindrical, and ciliated cells are found. The first come from the naso-pharynx; the others usually from the trachea and bronchi, but may come from the nose. Desquamated alveolar epithelium is difficult to demonstrate. The pigmented cells found almost wholly in chronic passive congestion of the lungs are chiefly, perhaps entirely, desquamated alveolar epithelium. The pigment appears as yellowish, yellowish-red, or brownish-red granules or as yellow diffuse pigmentation. Occasionally, however, it surrounds granules of carbon, and then appears brownish or grayish-black. The pigment is derived from the blood, and will usually give the iron reaction (see page 423), but very young or old pigment will not.

4. *Fatty Detritus.*—Fat-drops are frequently found, due to the fatty degeneration of cells.

5. *Elastic fibers* (Fig. 155) occur singly, but more often as a network. They are recognized by their sharp, dark outlines, due to their high degree of refractiveness, and by their marked degree of resistance to acids and alkalies by which

other like tissues, such as connective-tissue fibers, are destroyed. Elastic fibers are most abundant in the caseous masses above mentioned. When these masses cannot be found, the thicker portions of the sputum are squeezed between a slide and cover-glass or between two slides, and examined with a low power. The examination is rendered easier by mixing a little sputum with a 10 per cent. solution of caustic potash or soda. In certain cases it is necessary to mix together equal parts of the sputum and 10 per cent. caustic potash or soda, and to boil the mixture until the sputum is dissolved. The solution is then mixed with four

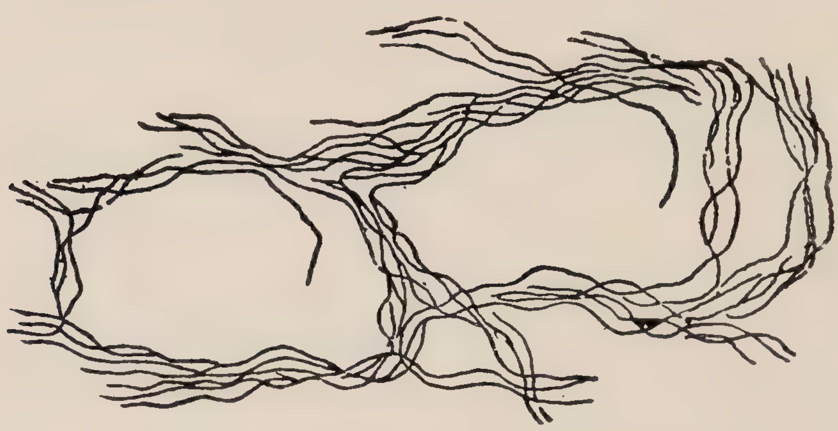


FIG. 155.—Elastic fibers (after Strümpell).

times its own volume of water and allowed to stand for twenty-four hours, when the sediment can be examined for the elastic fibers.

Vegetable and Animal Parasites.—Of the vegetable parasites, the most important is the tubercle bacillus (for its examination see page 184). Other bacteria sometimes examined for are the pneumococcus, the influenza bacillus, and actinomyces.

W. H. Smith's Method of Staining Bacteria in Sputum.—This has been found particularly useful in demonstrating the pneumococcus in the sputum. The sputum or other material should be fresh. The cover-glasses should be spread as thinly as possible and fixed by passing three times through the flame in the usual manner.

1. Stain in aniline-gentian-violet solution for a few seconds, gently warming until the staining fluid steams.

2. Wash in water.

3. Cover with Gram's solution of iodin for thirty seconds.

4. Wash with 95 per cent. alcohol until the color ceases to come out.

5. Wash with ether for a few seconds. (To remove fat.)

6. Wash in absolute alcohol for a few seconds.

7. Stain one to two minutes in a saturated aqueous solution of eosin.

8. Wash with absolute alcohol for a few seconds.

9. Clear with xylol.

10. Mount in balsam.

The pneumococcus is stained blue-black, while the capsule is stained pink. This method gives beautiful preparations. With the following modification it has been used by Smith as a routine stain for sputum. The advantage of this modification is that influenza bacilli and other bacteria which do not stain by Gram's method are clearly brought out, as are also eosinophilic leucocytes. This modification consists in washing the preparation with Löffler's alkaline methylene-blue solution just after it has been stained with eosin, as described above, and then, after the excess of eosin has been removed by the methylene-blue, steaming the methylene-blue solution for a few seconds while on the cover-glass. The preparation is then washed in water, rinsed with alcohol, cleared with xylol, and mounted in balsam.

Of the animal parasites, the entamœba histolytica is sometimes found secondary to an hepatic abscess which has perforated into the lung (see page 454). Portions of the membrane of an echinococcus cyst or the hooklets from the head may be found in the sputum, but infection with this parasite is very rare in this country.

Of the crystals which occur in sputa, the most important are the Charcot-Leyden crystals, found mainly in bronchial asthma, and the crystals of the fatty acids, of cholesterin, and of hematoidin. Tyrosin and leucin are much more rare.

The **Charcot-Leyden crystals** are colorless, elongated octahedra of varying size, soluble with difficulty in cold

water, insoluble in alcohol, ether, chloroform, and dilute saline solution.

Hematoidin crystals occur as ruby-red rhombic plates or columns.

Cholesterin crystals (Fig. 156) occur as the well-known small and large rhombic plates.

The **fatty-acid crystals** occur as long, pointed needles,

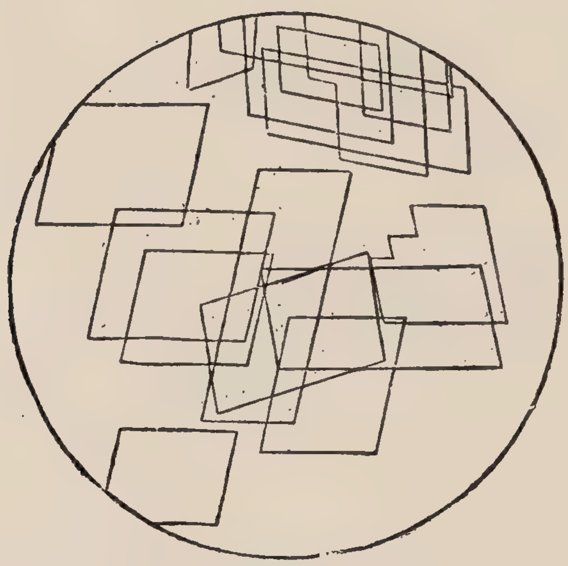


FIG. 156.—Crystals of cholesterin (after Strümpell).

either singly or in groups. They are easily soluble in ether or hot alcohol, insoluble in water and acids.

Examination of the Gastric Contents.—The microscopic examination of the contents of the stomach is much less important than the chemical. Fresh blood is easily recognized by the microscope. Disintegrated blood must be examined for chemically by the hemin test, as follows:

Mix a little of the suspected material with a crystal or two of common salt, or place it on the thin layer of salt formed by slowly evaporating a small drop of normal salt solution on a slide. Cover with a cover-glass, and run in enough glacial acetic acid to fill up the space between slide and cover. Warm the slide over a flame for three-quarters to one minute until bubbles arise, adding more glacial acetic acid as evaporation takes place, until a faint reddish-brown tint appears. Then let the acetic acid evaporate entirely, and run glycerin in from the edge of the cover-glass. Microscopic examination will show dark-brown rhombic plates or columns of hemin if blood is present.

Shreds of tissue or bits of mucous membrane are sometimes found in the vomitus or removed by means of a stomach-tube. Examination of them in the fresh condition, or, more satisfactorily, in stained sections after hardening and imbedding, will sometimes give definite information in regard to the condition of the mucous membrane, or render possible the diagnosis of a malignant growth.

Examination for Free Hydrochloric Acid.—Of the following tests, that with Congo-paper is the quickest and easiest, but shows only that a free acid is present. To prove that the free acid is hydrochloric acid the phloroglucin-vanillin test or one of the others is necessary.

1. *Congo-paper* is turned blue by free acids only. Free hydrochloric acid turns it of a cornflower-blue, a tint obtained with lactic acid only when in much greater concentration than is ever present in the stomach. Congo-paper is used simply by dipping it into the stomach-contents, preferably after filtration.

2. *Günzburg's Test with Phloroglucin-vanillin.*—The solution consists of—

Phloroglucin,	2;
Vanillin,	1;
Absolute alcohol,	30.

Three or four drops of this solution are placed with an equal amount of the filtrate from the stomach-contents in a porcelain dish and carefully heated over a small flame. Keep the dish in constant motion, and do not allow the mixture to boil, because boiling prevents the reaction from taking place. If free hydrochloric acid is present, a rose-red mirror is produced. The phloroglucin-vanillin solution does not always keep well, so that it is best to keep alcoholic solutions of phloroglucin and of vanillin in separate bottles, and to mix together one or two drops of each when required.

3. *Boas' Resorcin Test.*—The solution consists of—

Resublimed resorcin,	5;
Cane-sugar,	3;
Alcohol, 94 per cent.,	ad 100.

It is used in the same manner as the phloroglucin-vanillin test. A similar but more transient mirror is produced.

Töpfer's Dimethyl-amido-azo-benzol test is highly recommended by Simon as superior to the phloroglucin-vanillin test. "One or two drops of a 0.5 per cent. alcoholic solution is added to a trace of the gastric contents, which need not be filtered; in the presence of free HCl a beautiful cherry-red color develops, which varies in intensity according to the amount of free HCl present."

Examination of the Feces.—In examining for worms and their eggs it is often best to dilute the feces with water, and then to examine the sediment both macroscopically and under the microscope. (For Entamœbæ see p. 454). The other protozoa are best looked for in fresh slide preparations.

For the cholera vibrio see p. 188, for the typhoid bacillus see p. 170.

The membranous casts sometimes found in feces consist almost wholly of mucus, cylindrical epithelial cells, and leucocytes. Bits may be examined fresh, or the casts may be hardened and sections made and stained after imbedding in celloidin.

Examination of the Urine.—Only those points are mentioned which come within the province of the pathologist.

Of the animal parasites, the bilharzia, the hookworm, the echinococcus, and the filaria sanguinis hominis are the only important ones (see pp. 459, 465).

Of the vegetable parasites, tubercle bacilli and gonococci are the most common; actinomycetes are very rare.

New growths in the kidneys are accompanied with hemorrhage in less than half of the cases, while new growths in the bladder almost invariably give rise to it. Fragments from new growths in the bladder are rare. A diagnosis of malignant disease from cells only is impossible. Pieces of tissue which show on microscopic examination the characteristic structure of cancer or other neoplasm must be obtained in order to render a diagnosis possible from the pathological (but not from the clinical) standpoint.

Method of Preparing the "Bacterial Vaccines" of Sir A. E. Wright.—These "vaccines" are suspensions of definite quantities of bacteria killed by heat, in a 0.9 per cent. solution of sodium chlorid. The method here described is a modification of Sir A. E. Wright's method as used in the Pathological Laboratory of the Massachusetts General Hospital.

As profuse a growth as possible of the bacterium is obtained in a number of "slant" culture-tubes, three or four tubes usually furnishing a sufficient mass of bacteria for the purpose. All of the bacterial growth in the tubes is collected in a thick suspension in sterile 0.9 per cent. saline solution in a sterile test-tube. This test-tube is then drawn out with the aid of a blast-lamp to a small diameter some centimeters above the level of the fluid, and is set aside to cool. When cool, the drawn-out portion is sealed off in the flame and the tube is thoroughly shaken during some minutes. The sealed extremity is then opened and a few drops of the suspension withdrawn into a small dish or onto a block of paraffin for the purpose of later determining the number of bacteria in suspension, after which the tube is again sealed in the flame. The suspension is now ready for sterilization. This is done by keeping the tube fully submerged in a water-bath at 60° C. for from one and a half to two hours.

The determination of the number of bacteria per cubic centimeter in the sample withdrawn from the sealed tube is made as follows:

The first step in the process is to thoroughly break up the clumps of bacteria so that each bacterium, as far as practicable, is free and separate in the suspension. This may have been already accomplished by shaking the suspension in the test-tube, but if not, then the breaking up of the clumps may be effected with the aid of a capillary pipette about 1 mm. in diameter, prepared from a piece of glass tubing of about the diameter used for the ordinary medicine dropper. To this pipette is affixed a tightly fitting rubber bulb similar to that used on a medicine dropper, but of the best quality of rubber. The smaller end of the pipette must

be squarely broken off. The breaking up of the clumps is effected by repeatedly forcing the bacterial suspension in and out of the pipette by manipulation of the bulb, while the pipette is held perpendicularly against the surface of the glass dish or paraffin block in such a way as to bring as much as possible of the circumference of the smaller end in contact with it, thus leaving minute clefts which are small enough to cause the breaking up of the clumps as they are forced through. In this process the pipette is most conveniently held in such a manner that the bulb may be manipulated with the thumb and forefinger, while the remaining fingers grasp the body of the pipette and steady it against the surface of the glass dish or paraffin block. With some bacteria, for example, the gonococcus, this procedure is not sufficient to break up the clumps, and in this case the shaking of some cubic centimeters of the suspension with fine sterilized sand in a small tube is resorted to.

The next step is to determine the number of bacteria per cubic centimeter in the suspension. This may be done in either of two ways.

One way is to mix thoroughly equal quantities of freshly drawn normal blood, of a fluid which prevents the coagulation of the blood, and of the suspension; then in stained smear preparations of the mixture determine the ratio between the number of red blood-corpuscles and the number of bacteria. Assuming five million red blood-corpuscles to a cubic millimeter, the number of bacteria per cubic centimeter is readily determined.

This procedure is carried out with the aid of a capillary pipette provided with a rubber bulb like the pipette described above. The mark is made on the pipette 2 or 3 centimeters from its smaller extremity, and into the pipette, while grasped in the hand, as before described, there is drawn up to this mark successively the fresh blood, the anti-coagulating fluid, and, finally, the bacterial emulsion, a small amount of air being allowed to enter the tube after each measure of fluid, and the end of the pipette wiped after each taking. The contents of the pipette are immediately expressed onto a

glass dish or paraffin block, and the elements in the various fluids thoroughly mixed by drawing the mixture in and out of the pipette repeatedly. Smears are then prepared and stained with Wright's blood-stain, as in the case of blood-smears. The counting is done under an oil-immersion objective with an eye-piece, upon the inferior lens of which a square has been marked out about 9 mm. on a side, with a wax pencil. The number of red blood-cells and bacteria seen within this ruled square are counted in various portions of the preparation until 1000 red cells have been counted. The anti-coagulating fluid employed consists of 1.5 per cent. sodium citrate in 0.9 per cent. sodium chlorid solution.

Another way of determining the number of bacteria per cubic centimeter in the emulsion is to count the bacteria without staining in a chamber similar to the Thoma-Zeiss blood-counting chamber. This method has been devised by one of us, and is regarded as much easier of execution than the one above described. The chamber used is manufactured by Zeiss for counting blood-plates by the Helber method. It should be supplied with an especially thin cover-glass (No. 146, Zeiss' Catalogue) to permit the use of the high-power dry objective with which the counting is made. The chamber is ruled like the "Thoma-Zeiss blood-counting chamber," and the rulings have the same value, except that the chamber is 0.02 mm. deep instead of 0.1 mm. For counting, the suspension of bacteria is diluted and mixed with distilled water 1 : 200 with the aid of the red blood-corpuscle pipette of the "Thoma-Zeiss" apparatus. By a simple calculation it will be apparent that the product of the multiplication of the average number of bacteria per small square by 4000 million will be the number of bacteria per cubic centimeter.

When the heating of the suspension is finished, the small end of the sealed tube is broken and a "planting" made from the emulsion upon the surface of a blood-serum "slant" to test the sterility of the emulsion. Immediately after this a sufficient quantity of the emulsion is mixed with sterile 0.9 per cent. saline solution to give a dilute suspension of the

volume of 50 c.c. containing the required number of bacteria per cubic centimeter. This is done as follows:

A small flask containing 50 c.c. of 0.9 per cent. of saline solution, closed with a rubber nipple, and the whole sterilized, is previously prepared. The quantity of the suspension necessary to give the desired number of millions of bacteria per cubic centimeter in a volume of 50 c.c. of saline solution having been determined by calculation, this quantity is withdrawn from the flask by means of the sterilized hypodermic syringe, the needle of the syringe being plunged through the rubber nipple while the flask is inverted; then the calculated quantity of the suspension is drawn up into the syringe and injected into the saline solution by passing the needle through the rubber nipple as before. Following this, 0.15 c.c. of lysol is similarly injected into the flask through the rubber nipple, and, after shaking, this diluted suspension constitutes the vaccine. Before injecting it, it should have been proved sterile.

The vaccines prepared from the staphylococci are made up so as to contain 600 million staphylococci in each cubic centimeter, while those of other bacteria are made up to contain only 100 million.

The dose varies according to the circumstances of the case. The full dose of the staphylococcus vaccine is 600 million, while a full dose of other vaccines is 100 million. The injection is made subcutaneously, usually in the abdominal wall in men and between the shoulder-blades in women, these being readily accessible and less sensitive areas. In charging the syringe for the injection, the needle is passed through the rubber nipple with the flask inverted. Before doing this, the surface of the rubber nipple should be sterilized, either with lysol, or by plunging the nipple and neck of the flask into hot water for a few seconds.

Serum Diagnosis of Syphilis by Means of the Wassermann and Noguchi Reactions.[1]—The importance of the so-called Wassermann reaction in syphilis, aside from its scientific interest, lies in the fact that it affords, as

[1] Written by Drs. O. S. Hillman and A. M. Burgess.

far as we know at present, a method by means of which accuracy in diagnosis may be increased, treatment better regulated, and probably a more definite opinion as to prognosis arrived at. The true nature of the reaction is still undetermined. We know that it is a complicated biological reaction, based on the phenomenon of complement fixation; a brief consideration of the theory of this phenomenon will therefore be necessary before stating the methods of carrying out the test.

In the first place, a few terms that are constantly being used will be defined:

Complement (*Alexine*).—This is a substance which is found in all fresh sera; its activity is destroyed by exposure to heat at 55° or 56° C. for half an hour. Serum treated in this way is said to be inactivated, and can be reactivated by the addition of another serum containing active complement. The sera of various animals differ in their complementary activity and also in their fixability, which is another characteristic that is possessed by complement. Anti-complementary action is a property which develops in a serum on standing or which may be present to a certain degree at the time the serum is drawn. In selecting a serum for the Wassermann reaction it is best to choose one which has the greatest degree of activity and fixability. It has been found that guinea-pig serum fulfils these demands probably better than the serum of any other species.

Amboceptor.—This is a specific reaction product, which may be present in any normal serum, and which can be produced in the serum of an animal by repeated injections (immunization) of cells or substances (erythrocytes, serum, egg-albumin, etc.), for which it has no natural amboceptor. Amboceptors that are normally present in serum are called natural amboceptors; those which are produced as the result of artificial immunization are called immune amboceptors. Amboceptors are classified according to the particular substances employed in their production; for example, hemolytic amboceptors (also called hemolysins) are those that are produced by the injection of red blood-corpuscles

into an animal; bacteriolytic amboceptors (bacteriolysins) are produced by the injection of bacterial extracts. An amboceptor is specifically defined by prefixing the term "anti-" to the name of the particular species employed in its production; for instance, when sheep's erythrocytes are the immunizing agent, the amboceptor is designated as an anti-sheep hemolytic amboceptor.

Complement and amboceptor are the two factors necessary in the production of serum hemolysis. This can be demonstrated by a simple experiment, as follows: immunize a rabbit to human red blood-corpuscles by means of repeated injections, thereby producing in the rabbit serum an anti-human hemolytic amboceptor. If serum from such a rabbit is brought into contact with a suspension of washed human red blood-corpuscles, dissolution of the corpuscles or hemolysis will take place; if, however, the rabbit serum be heated to 56° C. for one-half hour (inactivated), and then corpuscles added, no hemolysis will occur. Finally, if normal human serum or normal guinea-pig serum be added to the mixture, hemolysis will go on as before. These three factors which enter into this reaction, namely, the complement, the hemolytic amboceptor, and the red blood-corpuscles, constitute what is called, for the sake of brevity, the hemolytic system.

The function of the amboceptor in the above reaction of hemolysis is to sensitize or prepare the erythrocytes for the action of the complement; the latter then has the power of causing dissolution of the red cells, resulting in a clear red fluid. Neither amboceptor nor complement acting alone can produce this result. For complete hemolysis a definite ratio must exist between the various factors—amboceptor, complement, and erythrocytes. The requisite strength and proportion of these three can readily be estimated by titration, which will be taken up under Standardization of Reagents.

Antigens and Antibodies.—Antigens are substances which, when injected into a suitable animal, are capable of producing in that animal substances called antibodies, the latter thus being specific reaction products. Erythrocytes, bacteria, and proteins are examples of antigens. Under antibodies are in-

cluded hemolytic and bacteriolytic amboceptors, agglutinins, and precipitins. Antibodies are also found in the serum of patients suffering from infections with micro-organisms. In typhoid fever, for instance, an antibody is developed in the patient's serum as a result of the action of the typhoid bacillus upon the immunizing mechanism of the body.

Generally speaking, it may be stated that antigens and antibodies bear a specific relationship toward one another; for instance, the hemolytic amboceptor produced by injecting a rabbit with sheep's red blood-corpuscles acts with these corpuscles only and with no others. The agglutination of typhoid bacilli by the serum of the typhoid patient is also an example of this intimate connection between antigen and antibody; this fact is made practical use of in the Widal reaction for the determination of the typhoid agglutinin (antibody). The phenomenon of precipitation is another instance of the visible and direct action between antigen and antibody. Both agglutination and precipitation are dual mechanisms requiring no intermediate agent to complete the reaction.

In syphilis an antibody is supposed to be developed in the patient's serum, probably through the action of the treponema pallidum. It seems to be doubtful, so far as we know at present, whether the antibody in syphilis is actually specific or not. However, from a practical standpoint it may be said that it is the presence or absence of this so-called syphilitic antibody that we seek to demonstrate in the serum diagnosis of the disease.

Complement Fixation.—As stated above, antigen and antibody unite with one another specifically, and, when united, acquire the property of fixing or absorbing complement. This fact can be best illustrated by the interaction of two sets of antigen-antibody combination. Take, for example, a suspension of typhoid bacilli (antigen) and bring it into contact with typhoid serum (antibody); if complement is now added, bacteriolysis will result. That complement has been fixed or absorbed by this antigen-antibody combination is evidenced by the fact that if red blood-corpuscles and their specific amboceptor (another antigen-antibody combination)

be added later, no hemolysis will occur; complement, in other words, is not available for hemolysis on account of being fixed by the first antigen-antibody combination. This is the well-known phenomenon of complement fixation or deviation of Bordet and Gengou, upon which the Wassermann reaction and its various modifications are based. The so-called syphilitic antibody present in a patient's serum when brought into contact with an antigen is capable of fixing complement. This reaction is indicated by absence of hemolysis when the other two factors of the hemolytic system are added.

When the reaction was first introduced, it was thought that the antigen used in the diagnosis of syphilis was specific, as it was then made from the liver of a syphilitic fetus. This was the nearest approach obtainable to actual extracts of the causative agent, namely, the treponema pallidum. During the past two or three years it has been conclusively proved that this original antigen is not specific, as it has been found that extracts of normal livers, as well as other organs, and also certain lecithin preparations, will fix complement in contact with not only luetic sera, but also sera from patients infected with leprosy, yaws, sleeping-sickness, and malaria. The variability in the statistics of different writers is probably due to the variety of antigens employed, and at present this appears to be the principal limitation to the specificity of the reaction. It does not seem advisable here to go into a discussion of the antigen question beyond mentioning the various extracts that are in common usage.

The aqueous extract of the liver of a syphilitic fetus, which is used as an antigen in the original Wassermann reaction, is not employed in the methods following on account of its instability, and, incidentally, on account of the frequent difficulty of obtaining syphilitic fetuses. The modifications of the original test which have been devised depend, for the most part, on variations in the source of the antigen and in the employment of a different hemolytic system. In the original reaction an antisheep hemolytic system is used. This method will be given in addition to Noguchi's modifica-

tion, in which an antihuman hemolytic system is substituted for the antisheep. In both methods, however, the same antigen is used, this being the acetone-insoluble fraction of an alcoholic extract of a normal organ (heart, liver, or kidney), as recommended by Noguchi. Plain alcoholic extracts of normal organs and of livers and spleens of syphilitic fetuses are used by many workers in the Wassermann reaction, but these probably do not possess so high antigenic properties as the acetone-insoluble fraction of the alcoholic extract. This, however, is a matter of opinion. In any case it is an easy matter to try out these different extracts in order to test their relative merits. It is believed that by using the acetone-insoluble antigen it is possible to get the maximum amount of antigenic power with the minimum of hemolytic and anti-complementary properties. This antigen has been in use with Noguchi's antihuman hemolytic system in the Pathological Laboratory of the Boston City Hospital for nearly two years, and has been used with Wassermann's antisheep hemolytic system for several months, giving in both methods satisfactory results. On account of its stability for long periods, this antigen is particularly valuable, especially when reactions are done at infrequent intervals. For purposes of distinction, the two following methods will be designated as the Wassermann and Noguchi reactions respectively, although, strictly speaking, the name Wassermann should be applied to the original method of doing the reaction—that is, with the aqueous extract of a liver of a syphilitic fetus.

In the Wassermann reaction the patient's serum is inactivated in order to destroy the native complement, which is present, as a rule, in an appreciable amount. Complement of known strength necessary for the reaction is supplied by fresh guinea-pig serum. In the Noguchi reaction an antihuman hemolytic system is employed, as Noguchi maintains that, owing to the presence in human serum of varying amounts of natural amboceptor for sheep's corpuscles, many positive reactions are rendered negative in the Wassermann test on account of an increase in the total amount of amboceptor present, thus disturbing the proper proportion between ambo-

ceptor and complement necessary for complete fixation. It is not necessary to inactivate the patient's serum in the Noguchi reaction, as the human complement is only very slightly hemolytic for corpuscles of the same species, and also because the amount of complement present is practically negligible, owing to the small quantity of patient's serum used for the test.

A detailed account of the apparatus needed, preparation and standardization of reagents, and technique of the two reactions follow.

Preparation of Reagents.—*Glassware.*—For the Noguchi reaction, small test-tubes 10 cm. in length and 1 cm. in diameter are suitable. For the Wassermann test-tubes about 14 cm. in length and 1.5 cm. in diameter should be used. Six or eight 1 c.c. pipettes, graduated to 0.01 c.c., and two or three 10 c.c. pipettes, graduated to 0.1 c.c., are required. There are also needed glass tubing for the storage of amboceptor serum and for making capillary pipettes, rubber pipette bulbs, and several large Petri dishes 15 cm. in diameter.

In performing the Noguchi and Wassermann reactions, absolute bacteriological asepsis is not required, but all tubes and pipettes should be thoroughly washed in cold water, *without soap or chemicals*, and dried for three-quarters of an hour in a hot-air sterilizer. Tubes in which serum is to be preserved for more than forty-eight hours should be thoroughly sterilized.

Centrifuge.—The centrifuge should be one of high sedimenting power, and should carry at least a four-tube head.

Water Bath.—A water bath (temperature, 37° C.) may be used instead of the ordinary incubator, in which case the time needed for all reactions and preliminary titrations is reduced one-half, owing to the more rapid warming of the tubes and their contents.

Saline Solution.—The strength of the saline solution to be used in the reaction is 0.875 per cent.—*i. e.*, 8.75 grams of sodium chloride c. p. to a liter of distilled water. It should be autoclaved before use.

1. Complement.—It is best obtained by bleeding two or

more guinea-pigs into a large sterile Petri dish and pipetting off the clear serum after several hours' standing at room temperature. The clot will yield still more serum on standing over night in the ice-chest. Complement deteriorates rapidly, and should preferably not be used when over twenty-four hours old; 0.02 c.c. of complement is regarded as the unit for use in Noguchi's method. For the Wassermann test, as here described, 0.1 c.c. of undiluted complement is used as the unit. The variations in the activity and fixability of the serum from different guinea-pigs make it advisable to use a mixture of serum from at least two animals.

2. *Amboceptor.—A. Antihuman.*—Both the rabbit and the guinea-pig furnish strong antihuman amboceptor, which easily activates guinea-pig complement. Of the two, the rabbit is preferable on account of its size. Noguchi has shown, however, that guinea-pig amboceptor is even stronger in activating guinea-pig complement than is that produced in the rabbit. Guinea-pigs, therefore, are to be preferred in case there is difficulty in obtaining human blood in sufficient quantities for the inoculation of rabbits.

Human corpuscles for injection may be obtained by defibrinating the blood when collected, or by adding to it 0.1 per cent. sodium oxalate to prevent clotting. (In using the latter method one part of a 1 per cent. solution of sodium oxalate should be added to nine parts of blood and the mixture thoroughly shaken.) The blood should then be strained through sterile gauze into sterile centrifuge tubes, the volume marked on the outside of each tube, and a large amount of saline solution added. After stirring the contents of each tube with a sterile glass rod they should be centrifugalized until the corpuscles have all sedimented. The supernatant fluid is then decanted or siphoned off and the washing repeated at least three times. Sufficient saline is then added to each tube to make its contents equal in volume to the original blood. The corpuscles are then ready for injection.

Rabbits should receive five intraperitoneal injections at intervals of from three to five days (preferably four). The following amounts of washed corpuscles should be used: 5,

8, 12, 15, and 20 c.c. Nine days after the last injection the animal should be killed and its serum tested for amboceptor. If guinea-pigs are used, four injections of 2, 3, 5, and 7 c.c. are given into the peritoneal cavity. The intervals are the same as in the case of rabbits.

The blood of the rabbit, nine days after the last injection, may be collected as follows: The animal is etherized, the carotid artery dissected out, and a thread passed beneath it; then, with a pair of sharp scissors, the artery is snipped half through and the blood allowed to flow into a large, sterile test-tube. It is kept at room temperature for several hours and then is placed on ice, and the clear serum is pipetted off every day for three or four days.

The different specimens of serum thus obtained are mixed and heated to 56° C. for half an hour, to destroy complement. After titration (see Standardization of the Amboceptor) the serum is hermetically sealed in glass tubes, which are to be opened as used. It should be kept in the ice-chest, or, better, frozen.

B. Antisheep.—Antisheep amboceptor is prepared in the same way as is the antihuman. As sheep's blood can ordinarily be obtained in sufficient quantities, rabbits are injected instead of guinea-pigs. Blood obtained at an abattoir can conveniently be collected and defibrinated in a sterile glass jar containing glass beads. The method of washing and the schedule for injection are identical with that already described.

3. Antigen.[1]—Bovine heart (which can be particularly rec-

[1] For the original Wassermann an aqueous extract may be prepared as follows: Take the liver of a syphilitic fetus and cut it up into fine pieces; mix one part with four parts of salt solution (0.85 per cent.), to which 5 per cent. carbolic acid is added in the proportion of 0.5 per cent. Shake the mixture in a dark bottle for four hours by means of a shaking machine. Centrifugalize the mixture and decant off the supernatant fluid. Keep in the refrigerator in a dark, rubber-corked bottle. After a few days a precipitate falls to the bottom of the bottle. The clear supernatant fluid is used as antigen.

An alcoholic extract of normal liver or heart, or of a liver of a syphilitic fetus, may be made as follows: The tissue selected is taken in the proportion of 1 gram to 5 c.c. of absolute alcohol. Cut the tissue up into small pieces and grind it with sand to facilitate extraction. Extract for five days at room temperature in an ordinary glass jar, shaking the jar occasionally. Allow it to

ommended) or human heart or liver is finely minced, weighed, and covered with ten times its weight of 95 per cent. alcohol. The mixture is placed in the incubator at 37° C. for a week, and is stirred or shaken vigorously once or twice a day during this time. The mixture is then filtered through filter-paper and evaporated to dryness at room temperature. This may be accomplished by exposing the liquid in large evaporating dishes to a current of air from an electric fan. The residue is dissolved in a large quantity of ether, the solution filtered, and the clear filtrate evaporated to dryness. This residue is again taken up with as small a quantity of ether as is needed to dissolve it. To the ethereal solution five volumes of acetone are added and the whitish precipitate is allowed to settle. Most of the supernatant acetone is then removed by decantation or siphonage, and the remainder is allowed to evaporate. A mass of sticky yellow to brown material remains. This mass is weighed and dissolved in a sufficient quantity of methyl alcohol to make a 3 per cent. solution. This is the stock antigen solution.

Measured amounts of this alcoholic solution may be kept hermetically sealed in test-tubes, which are to be opened as used. It is stable at room temperature. For use, one part of it should be combined with nine parts of normal saline, making an opalescent emulsion. Inasmuch as in this form the antigen is often not stable for more than two weeks, it is advisable to keep the alcoholic preparation sealed in small amounts. For this purpose small test-tubes, such as are used in the Noguchi test, are convenient.

4. Patient's Serum.—Blood may easily be obtained in most cases from an arm vein. For this purpose ordinary sterile hypodermic needles are suitable. After the arm has been scrubbed with soap and water and alcohol, a tourniquet is applied to the upper arm and the needle is inserted into a

stand about two weeks. Use the clear, supernatant fluid for antigen, diluting it to the extent desired with normal salt solution. In hot weather keep the jar in the refrigerator.

Whichever antigen is used, it must first be titrated to determine its strength and suitability.

distended vein. The blood is allowed to drop from the needle into a sterile test-tube. In this way from 5 to 15 c.c. can ordinarily be obtained with little difficulty. Blood may also be taken from the ear or finger.

The blood is allowed to stand at room temperature, and on separation of the clot the clear serum is pipetted off. If the clot adheres to the side of the tube, it should be gently separated by a sterile glass rod or platinum needle. Serum from a specimen to which sodium oxalate or other chemical has been added is unsuitable for the test.

5. Corpuscle Suspension.—The human corpuscle suspension may be prepared by washing human corpuscles, as described under preparation of the amboceptor, and making a 10 per cent. suspension. A simple method is to fill a graduated centrifuge tube with 9 c.c. of saline, drop in 1 c.c. of blood from one's own finger, wash twice, and make up to 10 c.c. with saline.

For the Wassermann test a 5 per cent. suspension of washed sheep's corpuscles is used. This is prepared by adding nineteen parts of saline to one of washed corpuscles (made up in volume to equal the original blood).

No corpuscle suspension should be used when over seventy-six hours old, or when there is any trace of hemolysis.

Standardization of Reagents.—*1. Amboceptor—Noguchi.*—Into two test-tubes put 0.4 and 0.1 c.c. of immune serum. Make the volume in each case up to 10 c.c. with saline. Next, to a series of ten small test-tubes add graded amounts from these tubes as follows:

Small Tubes.

Tube 1.—4 per cent. amboceptor	(1)	.4 c.c.	=	.016	undil. amboceptor serum.
	(2)	.3 "	=	.012	" " "
	(3)	.2 "	=	.008	" " "
	(4)	.1 "	=	.004	" " "
Tube 2.—1 per cent.	(5)	.3 "	=	.003	" " "
	(6)	.2 "	=	.002	" " "
	(7)	.1 "	=	.001	" " "
	(8)	.08 "	=	.0008	" " "
	(9)	.05 "	=	.0005	" " "
	(10)	.03 "	=	.0003	" " "

To all tubes add 0.1 c.c. of a 20 per cent. solution of fresh guinea-pig complement and 0.1 c.c. of a 10 per cent. suspension of washed human corpuscles. Make the contents of every tube up to 1 c.c. with saline. Incubate two hours. The smallest amount of amboceptor which has caused complete hemolysis at the end of this time is the amboceptor unit.

Each time the Noguchi test is performed the amboceptor should be retitrated, so that the unit may be accurately determined with regard to the complement and corpuscles actually in use. For example, if the titre (standard of strength) of the amboceptor was shown in the original titration to be 0.001 each time the test is performed, a titration such as the following should be carried out:

Amboceptor diluted to 1 per cent.

1. 0.15 c.c. 1 per cent. amboceptor.	+	1 unit of complement (0.1 c.c. 20 per cent. complement and 0.1 c.c. 10 per cent. washed human corpuscles) to every tube.
2. 0.12 c.c. 1 " "		
3. 0.1 c.c. 1 " "		
4. 0.08 c.c. 1 " "		
5. 0.05 c.c. 1 " "		

Make the volume of every tube up to 1 c.c. Incubate two hours. This can conveniently be carried out so that its incubation is finished at the same time as the first incubation of the test.

If it is preferred to use the amboceptor dried on paper, the following method is recommended by Noguchi: Cut filter-paper (Schleich & Schüll, No. 597) into squares 10 x 10 cm. and place in a large Petri dish. Pour over them the amboceptor serum (about 10 c.c. for ten squares), saturating as evenly as possible. Absorb any excess with more filter-paper. Separate as quickly as possible and dry on clean, unbleached muslin for five or six hours. Cut into strips 5 mm. in width. In this form the amboceptor may be titrated by adding various lengths to a series of tubes containing complement and corpuscles, as before. Noguchi recom-

mends using the amboceptor in this form without a preliminary titration each time the test is performed.

Wassermann.—The titration of antisheep serum may be carried out as follows: Make 4 per cent. and 1 per cent. solutions, as in the case of the antihuman serum. Then, to a series of large test-tubes, add the same graded amounts of the diluted serum (see Titration of Antihuman Amboceptor). To each tube add 0.1 c.c. of undiluted guinea-pig serum and 1 c.c. of 5 per cent. washed sheep's corpuscles. Make up the volume of each tube to 3 c.c. Incubate two hours. The unit of amboceptor is contained in the smallest amount of serum which has produced complete hemolysis at the end of this time. As in the case of the Noguchi reaction, a preliminary titration of the amboceptor should always be carried out in connection with the performance of the test.

2. *Antigen.*—The antigen emulsion, to be suitable for use, must not possess any appreciable *hemolytic* or *anticomplementary* qualities, and must possess sufficient *antigenic strength* to allow its use in conveniently small quantities.

1. Noguchi system.

The following tests are recommended by Noguchi:

Tube 1.

0.4 c.c. antigen emulsion.
0.1 c.c. 10 per cent. human corpuscles.
Incubate two hours.

Complete *absence of hemolysis* at the end of this time indicates that the antigen emulsion does not possess any appreciable *hemolytic property.*

Tube 2.

0.4 c.c. antigen emulsion.
0.1 c.c. 40 per cent. guinea-pig serum.
Make volume 1 c.c. with saline.
Incubate one hour.
Add – 2 units of amboceptor and 0.1 c.c. 10 per cent. corpuscle suspension.
Incubate two hours.

Complete hemolysis indicates that the antigen is not inherently *anticomplementary.*

Tube 3.

1 unit of syphilitic antibody.[1] 0.02 c.c. antigen emulsion. 0.1 c.c. 40 per cent. guinea-pig serum. Make up to 1 c.c. Incubate one hour. Add — 2 units of amboceptor and 0.1 c.c. 10 per cent. corpuscle suspension. Incubate two hours.	*Absence of hemolysis* shows that 0.02 c.c. of the emulsion has sufficient antigenic strength to fix completely two units of complement.

An emulsion which fulfils the above requirements is suitable for use. The dose to be used in the Noguchi test is 0.1 c.c. (at least 5 units).

2. *Wassermann System.*—The titration of the antigen for use in the Wassermann may be carried out in exactly the same way, using double the amount of the emulsion and the amounts of serum, complement, corpuscles, etc., recommended under "Technique of the Wassermann Test."

Technique of the Noguchi Reaction.—1. Set up a preliminary amboceptor titration (see Standardization of Amboceptor) and place in incubator.

2. Set up two rows of small test-tubes as follows:

(*a*) Two tubes (one in front row and one in back row) for every specimen of serum to be tested.

(*b*) A similar set for a positive control serum.

(*c*) A similar set for a negative control serum.

3. Add:

(*a*) To both tubes in every set 2 capillary drops of the serum to be tested in that set. (Of cerebrospinal fluid use 0.2 c.c.)

(*b*) To all tubes 0.1 c.c. of 40 per cent. guinea-pig serum.

[1] The unit of syphilitic antibody, so called, may be determined by ascertaining the highest dilution of a known positive serum, one drop of which will completely fix the complement in the ordinary test (see Technique of Test). A series of tubes containing various dilutions of the serum are prepared and one drop from each is tested. The antigen to be used in this preliminary determination should be an antigen of proved strength, or, if such is not to be had, 0.4 c.c. of the emulsion to be tested, which amount may fairly be estimated to contain an excess of antigenic property.

(*c*) To front row only, 0.1 c.c. antigen emulsion. Make the volume of every tube 1 c.c.

Incubate one hour at 37° C.

4. Add to every tube 2 units of amboceptor and 0.1 c.c. 10 per cent. washed human corpuscles.

Incubate two hours at 37° C.

At the end of two hours the results may be read.

Technique of the Wassermann Reaction.—1. Set up a preliminary titration of the amboceptor and place in incubator.

2. Set up two rows of large test-tubes as follows:

(*a*) A set of two tubes (one in front row and one in back) for each serum to be tested.

(*b*) A set for a positive control serum.

(*c*) A set for a negative control serum.

3. Add:

(*a*) To both tubes in every set 0.2 c.c. of the serum to be tested in that set. (Of cerebrospinal fluid use 1 c.c.)

(*b*) To all tubes 0.1 c.c. of undiluted complement.

(*c*) To front row only, 0.2 c.c. antigen emulsion. Make volume of every tube up to 1.5 c.c.

Incubate one hour at 37° C.

4. Add to all tubes 2 units of amboceptor and 1 c.c. of a 5 per cent. suspension of sheep's corpuscles.

Incubate two hours at 37° C.

At the end of two hours the results may be read.

Interpretation of Results.—The judgment of a positive or negative reaction depends upon the absence or presence of hemolysis at the end of the incubation period or within a few hours (two to five) after the tubes have been standing at room temperature. As a rule, the reactions that are going to be clean-cut can be read at the end of the incubation period.

There is no difficulty in deciding frank positive or negative reactions, but difficulty is sometimes encountered in interpreting the gradations that are not infrequently seen where varying degrees in the intensity of hemolysis are present. Certain points are to be observed with both methods before the reactions are finally read. These are as follows:

1. The back row of tubes should be completely hemolyzed at the end of the incubation period in order to make sure that the hemolytic system is working satisfactorily, and, also, that the sera which are being examined are not anticomplementary.

2. The negative control should show complete hemolysis in both front and back tubes for the same reasons.

3. The positive control should show no hemolysis in the front tube, where the antigen-antibody combination has occurred, but in the back tube hemolysis should be complete for the same reasons noted under "1."

These three conditions being fulfilled, the test reactions may now be read.

In the Wassermann test, positive reactions are indicated at the end of the incubation period by absence of hemolysis in the front tubes. When the tests are first taken out of the incubator (or water-bath), a pink to pinkish-gray cloud extends up from the bottom of the tubes and may take in almost the entire bulk of fluid present. This is due to suspension of a certain number of red blood-corpuscles in the fluid. After standing a couple of hours, however, the corpuscles settle out in a sharp layer at the bottom of the tubes which are strongly positive, leaving a clear, colorless, supernatant fluid. In the Noguchi modification a similar condition is observed in positive reactions, but owing to the smaller amount of corpuscles and fluid, settling takes place more rapidly than in the Wassermann tubes. Reactions are sometimes encountered with the Noguchi test, in which the corpuscles settle out in a sharp layer, but the supernatant fluid, instead of being perfectly colorless, is slightly red colored. These reactions, however, are to be regarded as positive, although not strongly so. Negative reactions by both methods result in a perfectly clear red fluid, with complete hemolysis and disappearance of all the red blood-corpuscles.

Weakly positive reactions are indicated in both tests by various degrees of coloring of the supernatant fluid, and by variations in the amount of red blood-corpuscles in the bottoms of the tubes.

In the Noguchi test, weak reactions are of special signif-

icance, as they give distinct information regarding the strength of the so-called syphilitic antibody; therefore, in reading these weak reactions, it is important to note the extent to which hemolysis has progressed. The Noguchi test is of particular value in cases that are undergoing, or have previously received, treatment.

The presence in human serum of variable amounts of natural amboceptor for sheep's corpuscles renders the Wassermann test less valuable as an indicator of the strength of the syphilitic serum.

In the Wassermann test, reactions which do not show complete absence of hemolysis are regarded as weakly positive; those which show well advanced hemolysis are called very weak or doubtful, and, if possible, these reactions should be done over again, using fresh patient's serum and carefully titrating the reagents.

From the foregoing statements it can be seen that in examining sera, the serologist should always be informed whether or not antisyphilitic treatment has been administered, in order that he may be able to interpret the results of the reactions with more value to the clinician.

It has been found helpful to send out with reports of Wassermann reactions a printed slip containing the following statements:

It has become customary to recognize three degrees of the positive reaction and to indicate them with one, two, or three plus signs. The interpretation to be placed on these different degrees of the positive reaction is as follows:

POSITIVE + is less than a 10 per cent. inhibition of hemolysis and should not be considered of any diagnostic value except in a case of syphilis under treatment; then it calls for further active treatment. (Some workers with the Wassermann test prefer to have the lowest degree of positive reaction include as high as 50 per cent. inhibition of hemolysis.)

POSITIVE + + is an inhibition of hemolysis, varying between 10 and 90 per cent. It should be considered as a positive diagnosis of syphilis only in connection with strong clinical evidence. In a case of syphilis which has been treated it calls for further active treatment.

POSITIVE + + + is complete inhibition of hemolysis, and is to be considered as a positive diagnosis of syphilis.

The Wassermann reaction is not absolutely specific for syphilis.

A positive reaction, not due to syphilis, is frequently obtained in yaws, trypanosomiasis, and leprosy, and according to some authorities may also be obtained in rare instances in other conditions, such as scarlet fever, malaria, lobar pneumonia, diabetes mellitus, and immediately following ether anesthesia.

A negative reaction is obtained in many cases of syphilis during treatment with salvarsan or mercury, and may occur in alcoholics.

Antisyphilitic treatment must be interrupted for two months before a negative Wassermann reaction can be considered to have any diagnostic value.

The blood to be tested by the Wassermann reaction must be used as fresh as possible, before hemolysis due to warmth or bacterial contamination has taken place. The blood can be kept in good condition for the test for twenty-four to forty-eight hours if placed in a refrigerator.

The Complement-fixation Test in Gonorrheal Infections.—The method and technique are essentially the same as that of the Wassermann test, except that the antigen is different.

Preparation of the Antigen.—The growth from twenty-four-hour cultures of the gonococcus on hydrocele agar slants is scraped off with a platinum loop and suspended in a 0.5 per cent. solution of carbolic acid in distilled water. The

proportion of cocci to fluid should be the entire surface growth on the ordinary slant culture in a three-quarter-inch test-tube to about 5 c.c. of the fluid. The suspension is to be kept at room temperature and to be shaken from time to time during a week, after which it should be heated to 56° C. for an hour and centrifugalized thoroughly. To the supernatant fluid is then added sufficient 9 per cent. sodium chlorid solution to make its content in that salt 0.9 per cent. This is the antigen. Every time it is used it must be titrated to determine its anticomplementary dose, and one-half of the largest amount which does not inhibit hemolysis is to be used in the test.

The suspension of the cocci in the carbolic acid water retains its fixing power for months, and it may be kept on hand in quantities as a stock from which, as needed, small quantities may be taken, centrifugalized, and the proper amount of sodium chlorid added.

Considerable variation in the fixing power of different antigens is observed, and it is advisable to use two or three antigens from different strains of gonococci in each test or a mixture of antigens from several strains.

The Complement-fixation Test in Echinococcus Infection.—The method and technique are the same as those for the Wassermann test, except that the cyst fluid, or an alcoholic extract of the cyst walls, is used as the antigen. The cyst fluid should be clear, and to it should be added carbolic acid in the proportion of 0.5 per cent. to preserve it.

The extract of the cyst walls is prepared by grinding them up with sand and extracting the mass with absolute alcohol, in the proportion of about one part cyst substance to five of alcohol, for several days.

The anticomplementary dose of the fluid or of the extract, the latter diluted 1 to 4 with normal salt solution, is to be determined by titration, and one-fourth of this quantity is to be used in the test.

These antigens are said to keep for months.

Lange's Colloidal Gold Test of the Cerebrospinal Fluid for Syphilis of the Central Nervous System.—

This account of the test is based upon the work of Dr. R. I. Lee and Dr. W. A. Hinton with it.

The test depends upon a change in the color of a solution of colloidal gold when it is mixed in certain proportions with the cerebrospinal fluid from cases of syphilis and certain other pathological conditions of the central nervous system.

For each test a row of 10 test-tubes, $\frac{3}{4}$ inch in diameter, is set up in a rack. Into the first tube on the left is measured with a small pipette 1.8 c.c. of a 0.4 per cent. solution of sodium chlorid, and into each of the other tubes 1 c.c. of the same solution. Into the first tube on the left is now measured 0.2 c.c. of the cerebrospinal fluid, and the contents thoroughly mixed by thrice drawing some of it high up into the pipette and expelling it. From this tube 1 c.c. of the fluid is withdrawn and transferred to the adjoining tube, the contents thoroughly mixed as before by means of the pipette, and 1 c.c. of this mixture then withdrawn and transferred to the next tube in the row, and so on. In this way half the contents of each tube is transferred to the next tube and mixed until the tenth tube is reached, when 1 c.c. of the contents of this tube, after thorough mixing, is withdrawn and rejected. In the row of 10 tubes there are thus obtained the following dilutions of the cerebrospinal fluid, starting from the left to right, namely, 1–10, 1–20, 1–40, 1–80, 1–160, 1–320, 1–640, 1–1280, 1–2560, 1–5120.

Into each tube is next measured from a 25 c.c. graduated pipette 5 c.c. of the colloidal gold solution or "reagent," which is described below, and the tubes are shaken.

The "reagent" should be of a color closely approximating "old rose" red, with the slightest blue or purplish nuance, and have a very slightly yellowish fluorescence. It should be clear and transparent.

With normal cerebrospinal fluid, free from serum or blood, no change of color should be apparent in the mixtures in any of the tubes. A single tube showing color change indicates faulty technique.

With pathological fluids, there is a change of the red color in two or more tubes to a more or less marked blue color

almost immediately. After twelve or twenty-four hours the color changes are more marked, and there is more or less deposit of a black sediment. After this period, in the tubes showing the more intense reaction, the blue color has become paler or the fluid may be colorless.

For recording the appearances in the various tubes six degrees of color change are to be recognized, and are expressed by numbers, namely: "±" representing the slightest increase in the bluish tint of the reagent, "1" a slightly greater change, and so on, "5" representing a colorless fluid. These color changes, if the technique is right, are gradual in the series, *i. e.*, there should be no marked difference between any two adjoining tubes.

Any form of syphilis of the central nervous system is indicated by varying degrees of color change; these changes are most marked in the third, fourth, and fifth tubes, counting from the left of the row, and corresponding to the dilutions of 1–40, 1–80, and 1–160 of the cerebrospinal fluid. Fluids from cases which have received intradural injections of salvarsanized serum may give reactions which are different from these and may be atypical. As a rule, the fluid of general paresis and cerebrospinal syphilis gives stronger reactions than that of tabes.

The fluid from cases of non-specific meningitis and brain tumor shows most marked color changes in the seventh and eighth tubes, corresponding to the dilutions of 1–640 and 1–1280; fluids contaminated by blood or serum may also produce color changes, most marked in these tubes. Bacterial contamination of the fluid weakens the reaction and may change a positively reacting fluid into a negatively reacting one.

The preparation of the colloidal gold "reagent" requires great care, and the obtaining of a solution which answers the requirements as to color and clearness appears to be dependent to some extent upon conditions beyond the control of the operator, so that several lots may have to be made up before one suitable for use is obtained. The directions for the preparation of the reagent are as follows:

Heat in a tall Jena glass beaker, of about 2 liters capacity, 500 c.c. of double distilled water to 60° C. The beaker is to be supported on wire gauze over a large Bunsen flame. When this temperature is reached, as shown by the thermometer, without removing the flame, run in 5 c.c of a 1 per cent. aqueous solution of yellow crystalline chlorid of gold and immediately afterward 5 c.c. of a 2 per cent. aqueous solution of potassium carbonate. Continue the heating until the temperature reaches 100° C., and until the smaller bubbles cease to be given off by the boiling fluid. Then run in 5 c.c. of a 1 per cent. aqueous solution of formalin, remove the beaker from the flame, and stir the fluid. The fluid should immediately, or within a minute, assume the "old rose" red color, and if suitable for use should have the characters above described. When cool it is ready to be used as the "reagent." If it is not perfectly clear when viewed in a test-tube of $\frac{3}{4}$ inch diameter, or if it is of a distinctly bluish tint, it should be rejected. It keeps for weeks.

Special Precautions to be Observed.—All glassware used must be chemically clean.

The water used for the "reagent," for making up the solutions and for the final washing of pipettes, flasks, beakers, etc., must be double distilled from Jena glass in a still free from rubber connections, and it must be kept in Jena glass flasks.

The cerebrospinal fluid must be free from blood and from bacterial contamination. Fluids kept bacteria-free have given the same reactions for weeks.

The small pipette used for measuring the cerebrospinal fluid and its dilutions must be cleaned with distilled water, absolute alcohol, and ether before use with each specimen of cerebrospinal fluid.

INDEX.

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